Forensics, phylogeography and population genetics: a case study using the Australasian snake-necked turtle, *Chelodina rugosa*.

By

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Statement of originality

The thesis is my original work and has not been submitted, in whole or in part, for a degree at this or any other university. Nor does it contain, to the best of my knowledge and belief, any material published or written by any other person, except as acknowledged in the footnotes, quotations or references.

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September 2008

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Statement of contribution

Because this thesis is written as a series of chapters prepared for publication in peer-reviewed journals, several people other than myself have contributed to the work, and they deserve acknowledgement. These include:

- Arthur Georges (Institute for Applied Ecology, University of Canberra), who provided guidance and supervision for all aspects of the PhD study, and assisted in the preparation of manuscripts.
- Nancy FitzSimmons (Institute for Applied Ecology, University of Canberra), who provided guidance and supervision for all aspects of the PhD study, and assisted in the preparation of manuscripts.
- James Robertson (Australian Federal Police), who provided guidance and supervision for all aspects of the PhD study, and assisted in the preparation of manuscripts.
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- Jane Hughes (Griffith University), who assisted in the preparation of the manuscript presented in Chapter 5.

These people are included as authors in the following chapters as well as the associated publications, in order of their contribution to the work. However, despite the collaborative nature of this thesis, the work within is my own, and I received no assistance other than that which is stated above.

I as primary supervisor agree with the above stated proportions of work undertaken for each of the published (or prepared for submission) peer-reviewed manuscripts contributing to this thesis:

Prof Arthur Georges Date

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Abstract

Illegal trade of wildlife is a serious and growing crime. One of the greatest challenges in international efforts for policing of the illegal wildlife trade is the provision of evidence. DNA technologies are ideal for providing evidence for wildlife crime because they can be used on degraded and highly processed products to address a wide variety of forensic questions (i.e. species, regional and population-level identification). Theory, techniques and principles from phylogenetics, phylogeography and population genetics provide the fundamental genetic data required for forensic applications. This thesis demonstrates the benefits of merging the disciplines of phylogenetics, phylogeography and population genetics into wildlife DNA forensics – an emergent field that uses DNA technologies to provide evidence for wildlife crime. A DNA forensic identification system was developed using the freshwater turtle *Chelodina rugosa* Ogilby, 1890 as a case study. This species was chosen because a commercial industry is established to supply the pet shop trade.

Application of conservation genetics to freshwater turtles and tortoises was reviewed. General areas where genetic principles and empirical data can be profitably used in conservation planning are identified. Monitoring trade and directing enforcement to protect overexploited turtle populations was identified as one of three crucial future directions for conservation genetics of freshwater turtles and tortoises.

The extent of illegal wildlife trade in Australia was examined using case prosecution data from the Australian Customs service for the period of 1994 to 2007. Of cases prosecuted, 46% were for attempted export and 34% for attempted import. Reptiles were the most targeted (43%), then birds (26%), and native plants (11%). For the majority of prosecutions (70%) the sentence was a fine (70%) that was consistently only a fraction of the market value. I argue that tougher penalties are required to deter criminals from engaging in illegal wildlife trade and initiatives for improved policing (such as DNA technologies) are urgently required.

DNA technologies that have been used to provide evidence for wildlife cases are critically evaluated. Emphasis is placed on the science that is required to form the foundation for forensic applications. Baseline genetic data for species, regional and population level identification of wildlife seizures can be provided by phylogenetic, phylogeography and

population genetic studies, respectively. I advocate greater collaboration of forensic scientists with conservation geneticists to develop research programs that will jointly benefit conservation of traded species and policing of wildlife trade.

Seventeen microsatellite markers were developed specifically for *C. rugosa*. Sixteen of the loci were polymorphic but three of these loci had null alleles. These 17 microsatellite markers were tested for amplification in eight other species with varying success; 98% amplification in *C. burrungandjii*, 72% in *C. canni*, 38% in *C. expansa*, 58% in *C. longicollis*, 67% in *C. mccordi*, 73% in *C. oblonga*, 81% in *C. parkeri*, and 68% in *C. pritchardi*. These microsatellite markers will be useful for population assignment, gene flow, mating systems and hybridization studies in the genus *Chelodina.*

Phylogeography of the Australasian freshwater turtle *Chelodina rugosa* was investigated using 867 bp of the mitochondrial control and ND4 regions. There were two major haplotype lineages for *C. rugosa* consisting of (i) Northern Territory and (ii) New Guinea and northern Queensland extending east to the MacArthur River. The designation of the New Guinea form as a distinct taxon (formerly called *C. siebenrocki)* was refuted. Extensive hybridisation between *C. rugosa* and *C. burrungandjii* in Arnhem Land were found by the mitochondrial analysis and 17 microsatellite loci. A hybrid between *C. rugosa* and *C. canni* was also confirmed. The mitochondrial gene trees and nuclear R35 gene tree (898 bp) were incongruent with respect to the phylogenetic relationships between *Chelodina sp.* (Kimberley) and *C. canni*. Further research using a suite of nuclear markers is required to resolve these phylogenetic relationships and the taxonomic status of *Chelodina sp.* (Kimberley).

Population genetics of *C. rugosa* in the Blyth-Cadel drainages of Arnhem Land was investigated to provide recommendations for their sustainable harvesting. There were no detectable impacts from traditional harvesting. Genetic diversity estimates were similar for harvested and unharvested populations. Levels of genetic structure in the Blyth-Cadel region were low and populations functioned as a metapopulation. I recommend that sustainable harvesting can be conducted, provided that the impacts of pig predation are alleviated and gene flow between sites, through natural or artificial means, is maintained.

A DNA-based forensic identification system for *C. rugosa* was developed. An 898 bp region of the nuclear R35 intron discriminated *C. rugosa* from all other Australian chelid turtles. Individuals with recent hybrids origins between *C. rugosa* and *C. burrungandjii* were identified by 17 microsatellite loci. Geographic sources of specimens could be assigned to three distinct regions by sequencing 867 bp of the mitochondrial DNA: (i) Darwin (Finnis basin), (ii) Arnhem Land, and (iii) eastern Queensland including southern New Guinea. Specimens could not be identified to a source locality at the population-level (using 12 unlinked microsatellite loci) in the Blyth-Cadel basin of Arnhem Land where a commercial trade has been established. Given the isolation and inaccessibility of the Arnhem Land region, this level of identification may be adequate to verify the legality of specimens from the commercial industry.

This thesis merges the disciplines of phylogenetics, phylogeography and population genetics with the growing field of wildlife DNA forensics. It highlights issues for the development of forensic identification systems for wildlife. Emerging technologies on the horizon, such as single nucleotide polymorphisms (SNPs) and pyrosequencing will herald a new era for wildlife forensics. They will complement existing technologies enabling rapid discovery of molecular markers and screening of wildlife seizures. DNA technologies will be an increasingly important tool in international efforts to fight the burgeoning illegal wildlife trade.

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Chapter 1 – General Introduction

Picture: The study species – Australasian snake-necked turtle *Chelodina rugosa* in its natural habitat – an ephemeral freshwater swamp in tropical northern Australia. Photo by Erika Alacs.

Chapter 1 – General Introduction

Wildlife crime and the utility of molecular technologies.

Illegal trade of wildlife is a growing and serious global crime worth more than US$20 billion per year (Interpol 2007). Organised criminal networks that deal in illegal drugs and arms have been linked to large scale smuggling operations of wildlife. These operations span many countries and jurisdictions (Cook *et al.* 2002; Warchol 2004). Australia is no exception. Australian nationals have been involved in wildlife smuggling syndicates spanning New Zealand, South Africa, South East Asia, Zimbabwe and the United States of America (Australian Customs Service 2004b; 2006a; 2007d). Illegal commerce of wildlife threatens the species targeted for trade, endangers biodiversity and creates a biosecurity risk for all countries involved. Exotic species introduced via illegal trade operations can establish in the wild and become pests or spread disease to native wildlife and agricultural species (Normile 2004; Pedersen *et al.* 2007; Reed 2005; Smith *et al.* 2006; Weigle *et al.* 2005). Detecting wildlife smuggling operations that are covert in nature and providing evidence that can be used to prosecute the perpetrators are the greatest challenges to curbing the burgeoning global illegal trade of wildlife.

Molecular technologies are ideal for the provision of evidence for wildlife crime (defined here and throughout as crimes that involve, or are committed against, wildlife) because they can be used on highly degraded and processed wildlife products to address a wide variety of forensic questions (such as the identification of seizures to species, sex, population, and individual levels) and are generally accepted as evidence for human crime by the forensic community (Bollongino *et al.* 2003; Dawnay *et al.* 2008; Gupta *et al.* 2005; Lorenzini 2005; Ogden 2008; Palsboll *et al.* 2006; Roman and Bowen 2000). While molecular technologies have been used routinely since 1985 for human crime (Gill *et al.* 1985; Jeffreys *et al.* 1985a; 1985b) their application to provide evidence for wildlife crime is still in its infancy. A short supply of molecular markers that are suitable for wildlife has limited their use for forensics to date. Molecular markers usually need to be developed specifically for each species of forensic interest and this can be an expensive and time consuming process. Complications often arise in the development of forensic systems for wildlife because of the sheer numbers of wildlife species. Each species has different mating systems, ecology, demography and evolutionary histories that influence the efficacy of DNA technologies for forensic application.

In developing DNA-based systems to provide evidence for wildlife crime, researchers also need to consider the provisions for accepting scientific data and theory as evidence that were defined by the landmark case of Daubert v Merell Dow Pharmaceuticals US 570 (1993). In this case it was stated that evidence must be based on the scientific method and should be: (i) empirically tested, in that the theory or technique must be falsifiable, refutable and testable; (ii) subjected to peer review and publication; (iii) known or potential error is quantifiable; and (iv) the theory or technique is generally accepted by the relevant scientific community.

Merging the disciplines of phylogenetics, phylogeography and population genetics into the field of wildlife DNA forensics.

Molecular technologies for the study of wildlife populations have been well established in the disciplines of phylogenetics, phylogeography and population genetics. Techniques employed in these disciplines can be empirically tested and some efforts are underway to quantify the errors associated with them when they are adapted for use in a forensic context (Baker *et al.* 1996; Budowle *et al.* 2005; Cassidy and Gonzales 2005), thereby meeting the provisions (i) and (iii) for acceptance as evidence. Mendelian genetics and Darwinian evolution theory form the foundation for phylogenetic, phylogeography and population genetic theories (Avise 2000; Avise and Wollenberg 1997; Bachmann 2001; Nei and Kumar 2000). These theories are generally accepted by the scientific community and appear in highly reputable peer-reviewed scientific journals, and thereby meet provisions (ii) and (iv) for acceptance as evidence. Hence, the theory and techniques from the disciplines of phylogenetics, phylogeography and population genetics are highly suited to provide evidence for wildlife crime.

In order to identify wildlife seizures to their species, region and population of origin, an understanding of the species boundaries and patterns of intra-specific and inter-specific diversity are required. Phylogenetic, phylogeographic and population genetic studies provide this baseline genetic information to form the foundation for forensic applications. Furthermore, the molecular markers used in these studies can be adapted for use in forensic identification systems of wildlife and thus greatly reduce developmental costs. Therefore, it makes sense to merge principles, theory and techniques from the disciplines of

phylogenetics, phylogeography and population genetics into wildlife DNA forensics, an emergent field that uses DNA technologies to provide evidence for wildlife crime.

The study species – *Chelodina rugosa*.

This project is part of a larger research programme funded by the Australian Federal Police (AFP) in partnership with the University of Canberra (UC) to develop DNA-based identification systems for Australian wildlife. Three PhD projects have been funded to cover major taxonomic groups including reptiles (this study), birds (current PhD study of Jo Lee) and marsupials (current PhD study of Linzi Wilson-Wilde). The freshwater Australasian snake-necked turtle, *Chelodina rugosa*, was chosen as the study species because a commercial trade has been established in Maningrida (Arnhem Land, Northern Territory) to supply hatchlings to the pet shop industry (Figure 1.1). The commercial enterprise was established in 2003 by the Bawinanga Aboriginal Corporation (BAC) to provide jobs for the local community that are compatible with their cultural values. To monitor trade of *C. rugosa* and protect the BAC industry it is important that *C. rugosa* from this industry can be distinguished from those that are illegally collected from the wild.

It should be noted that there is some confusion with regard to the designation of the study species as *C. rugosa* (Ogilby 1890). A recent study of Thomson (2000) found that the holotype of *C. oblonga* (Gray 1841) was actually a specimen of *C. rugosa* (Ogilby 1890). Because Gray was the first to describe the form in 1841 the name *C. oblonga* strictly takes precedence. However, since 1967 our study species has been erroneously but consistently referred to as *C. rugosa*. To avoid confusion and because an application has been made to preserve the current usage of the name *C. rugosa* to the International Commission of Zoological Nomenclature (ICZN) (Thomson 2006), the name of *Chelodina rugosa* is used for my study species throughout this thesis

Chelodina rugosa (Pleurodira: Chelidae) is a member of snake-necked turtles in the Australasian genus *Chelodina* characterised by exceptionally long necks that have evolved independently of the long-necked *Chelus* and *Hydromedusa* turtles of South America (Georges *et al.* 1998). The long neck confers benefit in the strike and capture of fast moving prey (Georges and Thomson 2006). *C. rugosa* is widely distributed in the wet-dry tropics of northern Australia extending from the Victoria River in the West to Princess

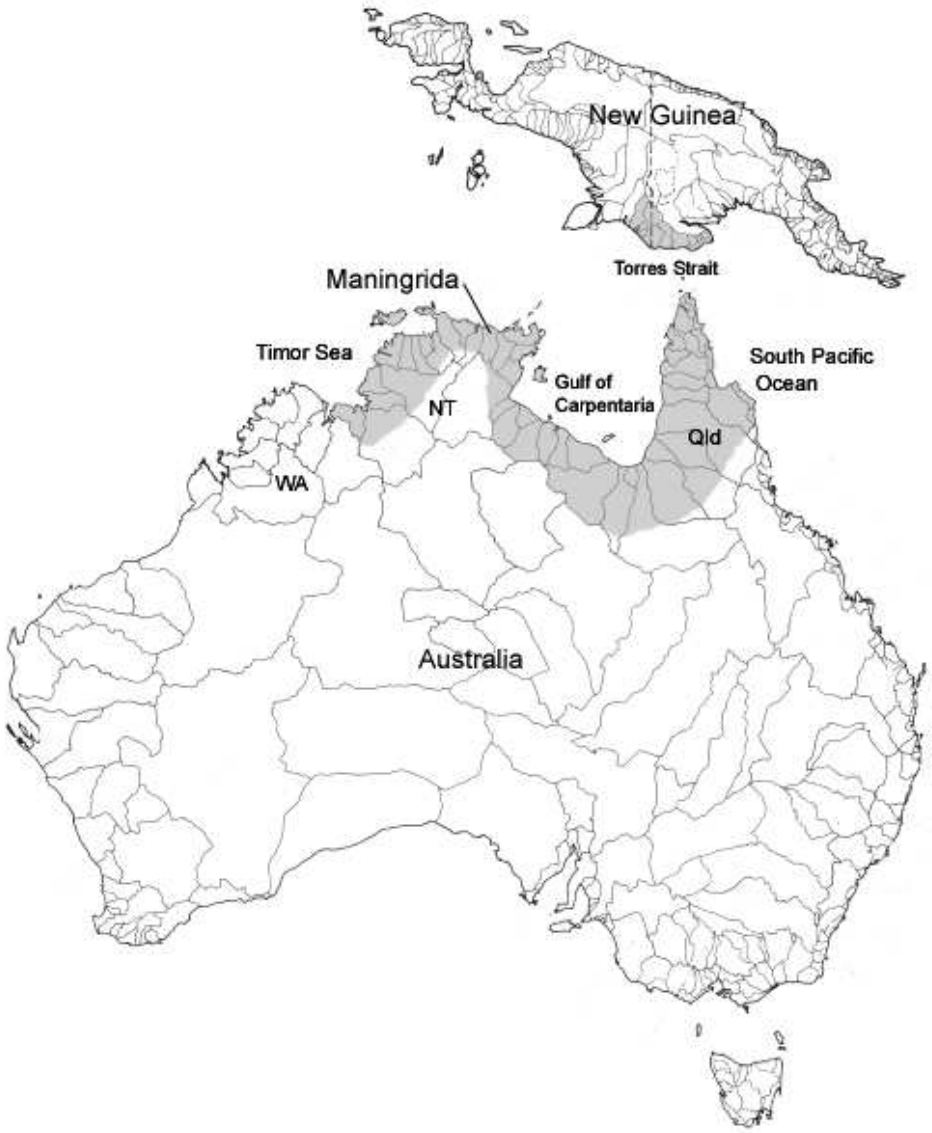

Figure 1.1 Distribution of the Australasian snake-necked turtle *Chelodina rugosa* (shaded) mapped onto the major river drainage basins of Australia and New Guinea. Maningrida is the township situated on the Blyth-Cadel River (Arnhem Land, Northern Territory) where a commercial industry in *C. rugosa* is established.

Charlotte Bay in the East. *C. rugosa* is also found in New Guinea from Merauke in West Papua (formerly Irian Jaya) to Balimo in Papua New Guinea where it was formerly known as *C. siebenrocki* (Rhodin and Mittermeier 1977). Throughout its range *C. rugosa* is locally abundant and attains high densities in freshwater ephemeral swamps, creeks and rivers (Cann 1998).The ecology of *Chelodina rugosa* has been extensively studied in the floodplains of the Darwin region (Kennett 1996; 1999; Kennett and Christian 1994; Kennett *et al.* 1993a; Kennett *et al.* 1993b; Kennett and Tory 1996) and Arnhem Land (Fordham *et al.* 2006a; Fordham *et al.* 2006b; Fordham *et al.* 2007a; 2008; Fordham *et al.* 2007b).

Thesis aims and structure

There are two parallel and overarching aims of this thesis. They are to (i) investigate the phylogeny and phylogeography of *Chelodina rugosa*, with particular attention to issues of taxonomic and conservation importance for *C. rugosa*, and (ii) demonstrate that the techniques, theory and methods in the disciplines of phylogenetics, phylogeography and population genetics can be adapted to provide evidence for wildlife crime. Merging of these disciplines into the field of wildlife DNA forensics will provide the baseline genetic data required for forensic applications, reduce overall costs in the development of molecular markers for wildlife, and provide data that meet the provisions to be accepted as evidence for prosecutions. A set of specific objectives are introduced in the following outline of thesis structure.

In Chapter 2, I present a broad overview of genetic issues in the conservation of freshwater turtles and tortoises. The objective is to identify critical areas where genetic data and concepts can be used for the conservation of freshwater turtles and tortoises. The techniques and concepts introduced in this chapter form a basis for later chapters directed specifically at *C. rugosa*. This chapter appeared in Chelonian Research Monographs.

In Chapter 3, I examine the extent of illegal trade of wildlife in Australia with a focus on wildlife that is imported into, or exported out of, Australia. Data from wildlife cases prosecuted by the Australian Customs Service for the period of 1994 to 2007 were analysed. The objectives were to (i) assess whether illegal trade of wildlife is on the increase, (ii) identify which taxa are targeted for trade, (iii) examine whether the penalties for convictions are an effective deterrent to criminals and, (iv) suggest future directions of

services to detect and provide evidence for wildlife crime in Australia. This chapter is soon to appear in print in Australian Journal of Forensic Sciences.

In Chapter 4, I describe and critically evaluate molecular technologies to provide evidence for wildlife crime. The objective is to assess the advantages and disadvantages of each molecular approach to provide evidence for wildlife crime. The importance of baseline genetic data for forensic applications is emphasised. This chapter is destined to be submitted to Forensic Science International, subject to amendment during the examination process for this thesis.

In Chapter 5, I present the results of a study to isolate and characterize microsatellite markers for *C. rugosa*. These microsatellite markers are tested for their utility in population genetic and hybridisation studies for *C. rugosa* and eight other species in the genus *Chelodina*. Informative microsatellite markers are used in later chapters for a population genetic study of *C. rugosa* (chapter 7) and to detect inter-species hybrisation and introgression between *C. rugosa* and its sympatric species (chapters 6 and 8). This chapter is soon to appear in Molecular Ecology Resources.

In Chapter 6, I re-assess the phylogeography of *C. rugosa*, relevant taxonomic issues, and inter-species hybridisation. The objectives are to (i) clarify whether *C. siebenrocki* of New Guinea should be regarded as a distinct or the same taxon as *C. rugosa*, (ii) identify phylogeographic breaks for *C. rugosa* and describe the historical vicariance events that may have shaped their current genetic diversity, (iii) clarify whether the Kimberley form of Western Australia, *Chelodina spp. (Kimberley)* should be regarded as a distinct or the same taxon as *C. burrungandjii*, (iv) examine the extent of hybridisation and introgression of *C. rugosa* with its sympatric species, *C. burrungandjii* and *C. canni*. This chapter has not yet been prepared for publication.

In Chapter 7, I present a genetic perspective on the sustainability of commercial harvesting of *C. rugosa* in the Blyth-Cadel River in the vicinity of Maningrida (Arnhem Land, Northern Territory). The objectives were to (i) evaluate the impacts, if any, of traditional harvesting on the retention of genetic diversity in populations, (ii) examine the population genetic structure of *C. rugosa* in the Blyth-Cadel River basin, and (iii) provide

recommendations to achieve sustainable commercial harvesting of *C. rugosa.* This chapter has not yet been prepared for publication.

In Chapter 8, I outline how the techniques, theory and data from phylogenetic, phylogeography and population genetic studies of *C. rugosa* can be adapted to develop an identification system to provide evidence for wildlife crime. Genetic data are drawn from Chapter 6 (phylogeography), Chapter 7 (population genetics), and used with additional data (phylogenetics) with the objective of developing a system to identify specimens of *C. rugosa* to species, regional and population levels. To reduce redundancy, methods that are covered in previous chapters are not elaborated in this chapter. However, for ease of reading the figures and tables from previous chapters that clearly illustrate the main findings are replicated. This chapter has not yet been prepared for publication.

Chapter 9 is my final synopsis that draws on each of the previous chapters and highlights their contributions to advances in science. Exciting new developments on the horizon for wildlife DNA forensics are discussed as well as their role in international efforts to curb the growing illegal trade of wildlife.

This thesis has been written as a series of papers. As outlined above, Chapters 2, 3, and 5 have been accepted for publication by peer-reviewed journals. These chapters appear exactly as they have been submitted except for the citation style that has been altered for consistency in the thesis. Chapters 4, 6, 7, and 8 are unpublished but have been prepared to require minimal re-working to be suitable for submission. To reduce repetition in the thesis for the unpublished chapters, redundant information has been removed wherever possible by reference to the appropriate chapters. Figures have been replicated if they improved the overall readability of these chapters. The research is my own, but as with any rigorous investigation, I have benefited from the invaluable contributions of my colleagues. My supervisors Arthur Georges, Nancy FitzSimmons and James Robertson were instrumental in the development of ideas, technical aspects of data collection, data analysis and writing up of results. Mia Hillyer provided assistance in the development and characterisation of microsatellite markers for *C. rugosa*. Damien Fordham provided most of the genetic samples for the population genetic study of Chapter 7. Contributions of several other colleagues were sufficient to warrant their co-authorship on papers. Co-authors are listed

under the title for each chapter in the reference to the publication. Others that contributed are listed in the acknowledgements.

Chapter 2 - Genetic issues in freshwater turtle and tortoise conservation.

Picture: A Malayan snail eating turtle (*Malayemys subtrijuga*) for sale in a popular pet market in Jarkarta, Indonesia. *M. subtrijuga* is a CITES Appendix II listed species and is threatened by the illegal pet trade. Photo by Erika Alacs.

Chapter 2 - Genetic issues in freshwater turtle and tortoise conservation.

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Abstract

Freshwater and terrestrial turtles are among the most imperilled biota on the planet, with over half of all extant taxa threatened with extinction. Active science-based management is required for the persistence of many species. Evolutionary genetic principles are often overlooked in the development of conservation and management plans, yet genetic data and theory can be critical to program success. Conservation biologists are encouraged to consider using genetic data and concepts when developing conservation strategies for turtles. We identify general areas where genetic principles and empirical data can be profitably used in conservation planning and provide empirical examples from the turtle literature. Finally, we suggest important areas for future research in chelonian conservation genetics.

Introduction

Turtles and tortoises are threatened globally. Approximately 63% (129 taxa) of 205 extant taxa are regarded as vulnerable or endangered, and many face extinction if effective conservation measures are not implemented. Widespread declines in numerical abundance and distribution documented in recent decades have been caused by habitat destruction, pollution, and overexploitation for trade in meat, pets, and traditional medicines (Gibbons *et al*., 2000; Moll and Moll, 2004; van Dijk, 2000). The number and intensity of pressures continue to mount, with climate change looming as a new threat, particularly for species with temperature-dependent sex determination (Booth, 2006; Miller *et al.*, 2004; Nelson *et al.*, 2002; Davenport, 1997; Janzen, 1994). Removal or amelioration of immediate threats does not necessarily ensure the persistence of endangered taxa or populations. Remnant populations are more often than not, small and highly fragmented, attributes that exacerbate their vulnerability to extinction from stochastic events and loss of genetic diversity (Lande, 1998; Hager, 1998).

Genetic diversity represents the raw material to facilitate adaptation to changing environmental conditions through natural selection. Hence, loss of genetic diversity can result in the loss of adaptive potential. Global environmental change is occurring at a rate unseen in the history of our planet (Hare and Meinshausen, 2006; Lenton, 2006; Li *et al.*, 2006). If chelonian species are to adapt and persist in the face of future changes, they will likely require active human intervention. Maintaining required levels of genetic diversity is only possible through conservation planning.

Knowledge of genetics is increasingly recognized as a critical element of conservation biology (Moritz, 1994; Soltis and Gitzendanner, 1999). Molecular techniques and methods of statistical analysis derived from evolutionary theory can be used to estimate how genetic diversity is apportioned spatially, how rapidly diversity will be lost over time, to identify crucial forces (anthropogenic or otherwise) contributing to present and future loss of diversity, to gain insight into fundamental aspects of an organism's biology, and to provide informed guidance for conservation and management (Moritz, 1999; Reed and Frankham, 2003; DeYoung and Honeycutt, 2005; Whiteley *et al.*, 2006). Despite the clear importance of genetics as a foundation for understanding turtle biology and directing turtle conservation actions, there is a paucity of turtle genetic studies relative to many other taxa.

We describe how population genetic theory and data can contribute to greater understanding of turtle biology and how this knowledge can be applied to achieve conservation objectives. We address eight major genetic issues that we believe are most relevant to turtle conservation -- 1) genetic diversity and potential for future adaptation; 2) genetic drift; 3) inbreeding and outbreeding; 4) selection; 5) gene flow and identification of management units; 6) clarifying taxonomy; 7) elucidating aspects of species' behavior and ecology; and 8) forensics. We provide a glossary of terms that are widely used in population genetics but may not be well known to biologists interested in turtles. Boxes are also included to emphasize several important concepts discussed in the text.

We have written the text to be accessible to the non-specialist. We have minimized the use of technical terms. Background theory and concepts are developed and empirical examples are presented to show relevance in areas of turtle conservation. We conclude by suggesting future priorities and directions. We advocate the use of genetics as only one component of a comprehensive conservation toolkit. Genetic principles and data should be

complemented with biological, ecological, zoogeographic, socio-economic and other relevant data in order to better direct decisions regarding chelonian conservation and management.

1) Genetic diversity and adaptive potential

Genetic diversity is a fundamental component of life on earth. Without it, there can be no evolution, no diversification, and thus, little or no biodiversity at any level of biological organization. In a contemporary sense, without genetic diversity populations cannot respond to biological or environmental changes through natural selection, be those changes natural or anthropogenic in origin (Frankham, 1995a; Frankham, 2005; Amos and Balmford, 2001).

The **phenotype** of an organism (its observable properties) is determined by the individual's genotypic composition, the expression of which is modified by the environment. Adaptation occurs when the phenotypic composition of a population shifts in response to environmental change. The new generation will preferentially represent the genetic composition of parents best able to cope with changes through their ability to survive and leave offspring. The resulting shift in genetic composition of the population reflects adaptation by **natural selection** (Orr, 2005). In the lifetime of an individual, responses to environmental change occur via **phenotypic plasticity** (non-heritable changes in phenotype such as faster growth when conditions are favourable). However, the capacity of an individual to be plastic also has a genetic basis. Variation is required at the level of **genes** coding for traits (Bradshaw, 2006; Via, 1993). Thus, phenotypic plasticity is itself an evolved trait. .

The rate of adaptive **microevolution** is roughly proportional to the **additive genetic variance**. Loss of genetic diversity is a fundamental concern in conservation biology because a populations' ability to evolutionary adapt to changing conditions is reduced when additive genetic variation is depleted (Amos and Balmford, 2001; Frankham, 2005). Given current rates of environmental change, the adaptive potential of populations will be critically linked to their probability of long-term persistence.

Levels of genetic diversity can be assayed by measuring variances and covariances in phenotypic traits among individuals. The field of **quantitative genetics** apportions

variation in phenotypic traits resulting from complex interactions between heritable genetic and environmental sources of variation. **Quantitative trait loci** (QTL) are the most relevant target of genetic studies of phenotypic adaptation (Falconer and MacKay, 1996; Lynch and Walsh, 1998; Barton and Keightley, 2002). However, quantitative genetic studies are difficult to conduct. Established pedigrees and/or large sample sizes are required to disentangle the effects of environment and **genotype** on quantitative traits (Barton and Keightley, 2002; Falconer and MacKay, 1996; Kirkpatrick and Meyer, 2004; Lynch and Walsh, 1998). It is often impossible to obtain large sample sizes from small wild populations, and establishing pedigrees is difficult and time-consuming. Small population sizes, long generation times, secretive mating habits, and the potential for long term sperm storage by females render turtles difficult subjects for quantitative genetic studies.

Genetic studies that employ **neutral genetic markers** are easier to conduct than quantitative genetic analyses. These two approaches differ because variation at neutral loci is presumably not subject to natural selection, but governed primarily by drift, **mutation** and migration (Holderegger *et al.*, 2006; Merila and Crnokrak, 2001). The adaptive potential of populations has frequently been inferred from population characteristics identified using neutral genetic markers, under the assumption that neutral and adaptive variations are positively correlated. Some empirical studies suggest that neutral markers can be predictive of variation at quantitative trait loci (Merila and Crnokrak, 2001), whereas other studies found no significant correlation (Reed and Frankham, 2001). The degree of correlation between the two measures of genetic variation will depend on the force of selection pressures on quantitative traits. Traits under the strongest local selection are expected to exhibit the greatest divergences from neutral variation. Traits that are not under selection will be largely shaped by the same microevolutionary forces as neutral regions (McKay and Latta, 2002). Neutral markers therefore must be evaluated carefully to infer adaptive variation. New emerging molecular technologies such as **genome**-wide scans will aid in development of measures of adaptive variation because these techniques can detect loci under selection in the absence of apriori knowledge of gene function (Kohn *et al.*, 2006; Storz, 2005; Schlotterer, 2003; Luikart *et al.*, 2003; Nielsen, 2005; see also McGaugh *et al.*, 2007).

An on-going debate in conservation biology concerns the relative importance of adaptive versus neutral genetic variation when weighing conservation options (McKay and Latta, 2002; Merila and Crnokrak, 2001; Holderegger *et al.*, 2006). **Heritability** measured for QTLs and **heterozygosity** (a measure of variation assayed using neutral molecular or biochemical markers) may both be related to current population **fitness** (Reed and Frankham, 2003). Thus, neutral genetic variation and trait heritability may both be useful as surrogates of population fitness and may be used to prioritise populations for conservation. The value of each approach for conservation and management of chelonians will be highlighted by brief discussion of two published examples.

Janzen (1992) estimated the **heritability** of pivotal temperature (T_{piv}) determining sex (i.e., the incubation temperature that produces a 1:1 sex ratio) for common snapping turtles (*Chelydra serpentina*). A standard quantitative genetic breeding design was not possible because *C. serpentina* takes around 10 years or more to reach reproductive maturity (Iverson *et al.*, 1997). Instead, eggs from 15 clutches were incubated near the T_{piv} for the population, such that the among-clutch variation in sex ratio could be interpreted statistically as quantitative genetic variation. Under controlled conditions, heritability of T_{piv} was estimated as 0.76 (possible range of 0 to 1) at 28 ^{o}C, suggesting substantial quantitative genetic variation for sex ratio. In nature, the temperatures of turtle nests may be influenced by the environmental conditions on the area of the nest (e.g. soil moisture, canopy cover, aspect etc.). When accounting for variations in the temperature of nests in a natural population of *C. serpentina* the *effective* heritability of T_{piv} reduced to 0.05, implying that genetic factors have a minimal effect on sex ratios compared to environmental factors.

Anthropogenic habitat alterations to nest thermal environments can greatly influence offspring ratios in turtles with temperature-dependent sex determination. Active management may be required to maintain equitable sex ratios for populations nesting in thermally-altered habitats.

Molecular and/or biochemical genetic markers can also provide estimates of levels of genetic diversity. Beheregaray *et al.* (2003) used two different neutral genetic markers (nuclear **microsatellites** and mitochondrial DNA **mtDNA**) to estimate levels of genetic variability within and among four island populations of Galápagos tortoises (*Geochelone*

nigra). Use of markers with different rates of mutation to new alleles facilitates estimation of the relative importance of contemporary vs. historical factors on population levels of genetic diversity. Microsatellites, with their faster rates of mutation, will illuminate the more contemporary situation compared to mitochondrial DNA (Avise *et al.*, 1992). Analyses of sequence variation in the mtDNA control region revealed long-term evolutionary divergence *among* populations on the four islands that was concordant with the geographic history of the region. Interestingly, for the island of Pinzón, there was evidence of historical population growth and retention of high levels of diversity (estimated from 10 microsatellite loci) *within* the population despite the populations' near extinction in the 1920s from predation by the introduced black rat. Survivors of the island population had maintained higher levels of genetic diversity than expected from population genetic theory. Hence, conservation efforts for these Galápagos tortoises may be best directed at retaining the relatively high existing genetic variability in two populations (Pinzón and La Caseta), and intensively managing to reduce further loss in two genetically depauperate populations (San Cristóbal and Cerro Fatal). Genetic studies as described above can be used to assess the merits of alternative management actions.

2) Genetic drift

Genetic drift arises from chance fluctuations in **allele** frequencies from one generation to the next. Even if individuals mate randomly within populations, changes in **allele frequency** will occur each generation. Due to chance alone, not all alleles will be present in the next generation, because not all individuals will successfully reproduce. Genetic drift is often described as a 'sampling effect' in which individuals produced in each generation represents a sample of the alleles in the ancestral **gene pool** of previous generations. Genetic drift is greater in smaller relative to larger populations (Nei *et al.*, 1975). For example, assume on average 70% of a turtle population is at a reproductive age. Not all sexually mature individuals will produce progeny for a given year for a variety of reasons, such as not finding a mate, poor nest site choice, predation of eggs, etc. Hence, effectively, only a fraction of the population will contribute genetically to the next generation and represents the **effective population size** (see Box 1). If the effective population size is small, then there is a greater chance that the "sample" will diverge in allelic composition from that of the overall gene pool. Thus the allele frequencies in the gene pool will drift.

Box 1: Calculating effective population size

The effective population size is the number of individuals in an "ideal" population having the same magnitude of random genetic drift, or loss of genetic diversity, or increase in inbreeding as observed. Effective population size is often less than the total population size due to the fact that not all individuals contribute equal numbers of progeny to the next generation. Effective population size can be estimated either using population genetic data or demographic parameters.

N_e estimated using demographic data-- If the number adult males and females is known, effective population size can be estimated as:

$$N_e = 4N_mN_f/(N_m+N_f)$$

where N_m and N_f are the number of breeding age males and females respectively . This equation defines the probability that 2 randomly selected genes in the current generation are copies of the same parental gene.

N_e estimated using empirical genetic data.-- Population allele frequencies change over time as a function of N_e and elapsed time in generations (t). Over small time intervals ($t<<2N_e$), and assuming that changes in allele frequency are due to drift, the expected variance in allele frequency [E(Fc)] is approximately $t/(2N_e)$. Using adults for a species which exhibits discrete non-overlapping generations, Waples (1989) defined the variance in allele frequency (Fc) between the 2 samples, which can be estimated for each locus as:

$$Fc = \left(\frac{1}{k}\right)\sum_{i=1}^{k}\frac{(x_i - y_i)}{(x_i + y_i)/2 - x_iy_i}$$

where x_i and y_i are the allele frequencies of the i^{th} of k alleles for adults in time periods t and t+1, respectively. Thus, Fc can be used to estimate N_e. Fc (variance in population allele frequency) must be estimated by Fc' (variance in sample allele frequency), which is also affected by random sampling errors in computing sample allele frequencies. Effective population size can be estimated by incorporating the variance in allele frequency due to the finite population size (genetic drift) and due to variation as a function of the finite number of samples used to estimate allele frequencies.

$$N_e = \frac{t}{2F_c - 1/(2S_o) - 1/(2S_t) + 1/N]}$$

where S_0 and S_t are the number of individuals samples in generations 0 and t. We can also estimate the effective number of breeders (not effective population size) using parent-offspring data (i.e., where t=1). This number can be adjusted to estimate effective population size. For example, for anadromous salmonids, Waples (1989) has shown that $N_e \sim gN_b$ where N_b is the number of breeders and g is the generation length (or average age of breeders) in the adult breeding population. With overlapping generations (i.e., breeding adults of several age classes contributing progeny to the next generation), estimating expected genetic drift becomes more difficult. N_e as defined above based on the temporal method must be corrected based on estimates of age-specific fecundity and survival .

If population numbers decline dramatically (i.e., the population experiences a **bottleneck**) or sex ratios become heavily skewed, or variance in male or female reproductive success is high, the effective population size (Ne) will be small and the probability that offspring represent a random sample from the original gene pool will be low. As a consequence of low Ne, alleles will be lost, particularly those present at low frequencies. When few alleles are present in the gene pool, opportunities for **heterozygous** combinations of alleles at a **locus** are reduced, and overall diversity will decline with each successive generation (see Box 2 for more detail). The rate of loss of diversity in a bottlenecked population depends on several related factors, including population size, severity and duration of the bottleneck, generation time, and gene flow (Allendorf, 1986; Richards and Leberg, 1995; Newman and Pilson, 1997; Garza and Williamson, 2001; Hedrick and Miller, 1992).

Kuo and Janzen (2004) used **neutral genetic markers** to compare the genetic diversity of a small, isolated population of imperilled ornate box turtles (*Terrapene ornata*) to that of a large population located within the main range of the species. Theory predicts that the small population size of the isolated population should over time lead to reduced genetic diversity due to the effects of genetic drift, relative to the large population. Genetic diversity was assessed using 11 polymorphic, nuclear **microsatellite** DNA loci for ~75 turtles from each population. Contrary to expectations, measures of genetic diversity did not differ between the two populations. However, the small population had a genetic signature that indicated a bottleneck in population size (that had occurred based on theoretical expectations). Why was there no detectable difference in levels of genetic diversity between populations differing in current numerical abundance despite a bottleneck persisting for 100-200 years?

Ornate box turtles have a relatively long lifespan, living on average 22 years in the wild (Metcalf and Metcalf, 1985). This longevity, long generation times, and overlapping generations are life-history traits characteristic of turtles that might retard the negative effects of drift on population levels of genetic diversity. The long duration of the bottleneck spanning hundreds of years (and several generations) may have also influenced the retention of genetic diversity. Short, but severe bottlenecks were found by England *et al.* (2003) to have a greater impact on loss of alleles than bottlenecks of lower severity occurring over several generations.

Not all turtles have retained high levels of genetic diversity after experiencing population bottlenecks. Similar to the ornate box turtle, the gopher tortoise, *Gopherus polyphemus*, in the south-eastern United States has suffered a bottleneck persisting for more than a century due to habitat destruction of favored longleaf pine forests, *Pinus palustris,* and harvesting of turtles for food. Populations were reduced numerically by up to 80% (Auffenberg and Franz, 1982). Schwartz and Karl (2005) estimated levels of genetic differentiation among and diversity within of gopher tortoise populations in Florida and Georgia using nine microsatellite loci. Genetic divergence amongst populations in both regions were high (average pairwise F_{ST} of 0.37 ± 0.17 and 0.14 ± 0.05 among Florida and Georgia populations, respectively). Values of F_{ST} greater than 0.10 are considered to be high (Wright, 1969) indicating restricted migration or **gene flow** (see section 5 and glossary). Populations which are reproductively isolated, for example within highly fragmented landscapes are more susceptible to loss of genetic variation due to drift.

Box 2: Predicting the loss of genetic diversity in populations from drift.

Expected loss of genetic diversity from the effects of drift, as measured by heterozygosity, can be predicted based on the population size. Population measures of heterozygosity can be measured as the proportion of individuals heterozygous at a locus. The expected proportion of original heterozygosity remaining after a generation of drift is [1-1/2N]. If population size remains constant over many generations the heterozygosity after t generations (H_t) can be estimated as:

$$H_t = (1\text{-}1/2N)^t H_o$$

where H_o is the population heterozygosity in the present population, and N is the adult breeding population size.

Population size and stochastic changes in allele frequency due to drift also have demonstrable effects on other population measures of genetic diversity such as the number of alleles per locus. Consider a diploid locus with n alleles present in frequencies p_1, p_2, p_3……p_n. The expected number of alleles remaining after a single generation (n') of random mating by N adults is:

$$E(n') = n\text{-} \sum_{i=1}^{n} (1 - pi)^{2N}$$

The probability that an allele will be lost is a function of the frequency of the allele in the population. Thus, alleles at greatest risk of loss are those that are rare (Allendorf 1986).

Founder effects have been well documented, where newly established populations have substantially reduced levels of genetic variance compared to sources (Hedrick *et al.*, 2001; Leberg, 1992). For example, only a small proportion of animals in the captive breeding programme of Galápagos tortoises (evaluated for 15 microsatellite markers) contributed to the repatriated population on the island of Española (Milinkovitch *et al.*, 2004). Variance in adult contributions can be attributed to several factors, most likely acting in concert, such as unequal access to mates, variance in fertility, unequal sex ratios, and differential survivorship of offspring. Re-evaluation of the breeding adults to equalize contributions of breeders will ensure that diversity is not compromised in the supplemented island population by the 'sampling effects' (Ramirez *et al.*, 2006; Sigg, 2006).

3) Inbreeding and outbreeding

Matings can occur between relatives, even if mating occurs at random and the population size is large. Inbreeding can have severe genetic consequences. The probability of matings between relatives will increase when populations are small in size, particularly if population size remains small over several generations, and in the absence of behavioural mechanisms to preclude inbreeding such as kin avoidance during mate selection. The primary effect of inbreeding is to change genotypic frequencies in favor of homozygous **genotypes** (see Box 3). Inbreeding can also lead to decreased fitness (**inbreeding depression**) due to the expression of **deleterious recessive alleles** through matings with close relatives. Inbreeding depression and the loss of heterozygosity probably contribute to many components of phenotype and fitness, including metabolic efficiency, growth rate, reproductive physiology, and disease resistance (Gilpin and Soule, 1986). The detrimental effects of inbreeding in captive (Ralls and Ballou, 1983) and natural populations (Keller and Waller, 2002) are widely accepted.

Population risk of extinction is related to population intrinsic rate of increase (Lande 1988). Declines in reproductive output and survival (the basic components affecting population growth) increase proportionally with levels of inbreeding (Falconer and MacKay, 1996). There is a considerable literature from case studies on captive populations (Lacy, 1997), laboratory populations (Frankham, 1995b; Reed *et al.*, 2002), natural populations (e.g. Frankham, 1997; Crnokrak and Roff, 1999; Keller and Waller, 2002), and from meta-analyses (review in Frankham (2005) and population viability simulations (Brook *et al.*,

2002) that document the negative impact of inbreeding depression and loss of genetic diversity onto probabilities of population persistence.

Inbreeding can be a major concern in natural and captive populations of turtles, particularly if populations are small and there is little or no exchange among populations. For many populations, exchange of individuals and genes among populations is becoming infrequent or impossible due to habitat fragmentation and human development creating impenetrable barriers to gene flow (see section 5). Isolated populations of turtles are at high risk of loss of genetic diversity through drift and inbreeding. Since adults of many species are long-lived and have reproductive life spans extending over long periods of time, there is the potential that they could mate with their sons and daughters, even grandsons and granddaughters, as adults. If there are no mechanisms to prevent mating with close relatives (i.e., kin recognition), inbreeding would accelerate loss of genetic variability and could result in expression of lethal recessive alleles leading to lower probabilities of population persistence. Levels of inbreeding will accrue in captive populations with high probability, so considerable attention has been devoted to design of captive breeding programs (Ebenhard, 1995; Philippart, 1995; Miller and Hedrick, 1993; see also Syed *et al.*, 2007).

Box 3: Estimating inbreeding in populations.

There are numerous definitions and ways to estimate inbreeding (reviewed in Templeton and Read 1996). At the population level, inbreeding (F) is a measure of deviation from random mating (Hardy-Weinberg). Population levels of inbreeding can be quantified empirically using molecular or biochemical markers by estimating the excess or deficiency of observed heterozygosity (H_o) relative to heterozygosity expected if populations were mating at random (i.e., under Hardy-Weinberg). For example, expected heterozygosity (H_e) for a locus with 2 alleles with frequencies p and q = (1-p) would be 2pq. F can be estimated as:

$$(H_e - H_o) / H_e \Longrightarrow 1 - (H_o/2pq)$$

Thus, if F is a measure of the proportional deviation of observed from expected heterozygosity,,, observed heterozygosity can be expected to diminish as

$$H_o = 2pq\ (1 - F)$$

and the frequency of homozygous and heterozygous genotypes in the next generation can estimated as:

Genotypes	AA	Aa	aa
Hardy Weinberg frequencies	p^2	2pq	q^2
Frequencies with inbreeding	$p^2 + pqF$	$2pq(1 - F)$	$q^2 + pqF$

One way to avoid inbreeding is to **outbreed**. The opposite of inbreeding depression is outbreeding enhancement, which is often referred to as **heterosis** or hybrid vigor (Lerner, 1954). Individuals from different populations are not likely to be homozygous for the same recessive alleles. Thus, outbreeding among individuals from different populations (wild or captive) can lead to masking of different **deleterious recessive alleles** present in different populations. If offspring from outbred matings subsequently contribute reproductively in future generations, and if the deleterious recessive alleles are present in low frequency, then these alleles are likely to be randomly lost from the population after several generations due to simple **Mendelian segregation** and genetic drift. The fitness of individuals and the long-term viability of an outbred population can be higher than that of either parental population due to the reduced frequency of these deleterious recessive alleles.

Box 4: Outbreeding depression causes a breakdown in co-adapted gene complexes.

Consider an outbreeding situation demonstrated using two loci. One locus has two alleles (A and a) and the second locus also has two alleles (B and b). There are two populations living in two different environments.

	Pop1	x	Pop2	F1 progeny	Progeny in later generations
Locus 1	AA	x	aa	Aa	AA or Aa or aa
Locus 2	bb	x	BB	Bb	BB or Bb or bb

Individuals in population 1 have 2 locus genotypes AA/bb whereas individuals in population 2 have genotypes aa/BB. If individuals from both populations inter-breed, offspring (F1 progeny) would all be Aa/Bb. The mixing of new alleles within the genetic background that has evolved within the environments inhabited by population 1 and population 2 can lead to problems. In the first generation, we may indeed see an increase in population fitness. If alleles A and B are primarily dominant to alleles a and b, then either AA or Aa genotypes or BB or Bb genotypes will still express the same phenotype. The initial reductions in the frequencies of homozygous recessive genotypes through outbreeding may actually be beneficial. However, expectations are that reductions in population fitness would be seen in later generations, where through Mendelian segregation, potentially maladaptive multi-locus genotypes (e.g., AA/BB, aa/bb) are present in the population.

Outbreeding up to some threshold level (i.e., perhaps between individuals from lineages of divergent populations) would be expected to result in increased population mean fitness. If such a simplistic perspective were indeed true, one universal conservation prescription for turtle populations of conservation concern would be to advocate mating individuals from

different populations. However, while inbreeding is essentially a concept formulated on a single locus basis, we need to consider outbreeding in the context of the entire **genome**. Declines in fitness can be realized over a much broader spectrum of outbred mating scenarios.

The phenomenon of **outbreeding depression** can be expressed in several ways. Under one scenario, declines in fitness for hybrids or outcrossed genotypes can occur due to "genetic swamping" of locally adaptive genes through gene flow or directed matings from another population that evolved under different ecological settings. We can consider two genotypes AA and BB that evolved in environments 1 and 2, respectively. AA has higher fitness in environment 1 than the BB genotype. Conversely, genotype BB has the higher fitness in environment 2. Hybrid genotype AB is not well adapted to either environment. The presence of inferior hybrid genotypes as a consequence of gene flow and subsequent reproduction will result in decreased population fitness.

The second way in which outbreeding depression can occur is by the breakdown of physiological or biochemical compatibilities between genes that have evolved in different populations. Interactions among alleles at several loci (**epistasis**) collectively affect fitness. Organisms have evolved in the context of specific environments and have evolved suites of genotypes across many genetic loci that are co-adapted to each environment. If new alleles are introduced via gene flow into the genetic background of the resident population, a loss in fitness may result from physiological or biochemical incompatibilities introduced through disruption of these co-adapted gene complexes (see Box 4). The fitness of the entire population could be compromised because outbred progeny are maladapted to either parental environment.

Outbreeding depression and inbreeding depression can occur simultaneously in a population. Fluctuations in population size and gene flow (either natural or directed) of maladaptive alleles can result in inbreeding or outbreeding depression, respectively, in natural populations, potentially reducing population fitness. Ultimately, in the design of breeding strategies, one must weigh the effects of potential past inbreeding in the population (which may have purged some deleterious alleles) relative to the effects of outbreeding on locally adaptive genotypic combinations. For many species of turtles, populations are numerically depressed, and in some cases, the species is only represented in

captive populations, potentially represented by few individuals originating from geographically different locales, or even from different taxonomically recognized subspecies or **evolutionarily significant units**. Decisions to breed across genetically and ecologically differentiated groups must weigh the potential detrimental consequences of both inbreeding and outbreeding to probabilities of species persistence.

4) Selection

Natural selection acts on the phenotypic composition of a population, altering it via the differential survival and reproduction of individuals (Lande and Arnold, 1983). Phenotypes that are better adapted to their environment (i.e., individuals with greater 'fitness') will be preferentially transmitted to the next generation. When the characters under selection have a genetic basis and are inherited, natural selection may result in the differential success of genotypes passing gametes to future generations (Nielsen, 2005). Selection can be decomposed into components, by taking a cohort born at the same time and following changes in the phenotypic and/or genetic characteristics of this cohort through each stage of the life cycle. Selection components include *viability selection* (differential survivorship), *sexual selection* (differential mating success), and *fertility selection* (differential production of offspring).

Selection may be introduced by humans through environmental changes to biotic and abiotic features. In captive populations, selection may be intentional such as a deliberate selection program designed to change some characteristic of the population. Selection can also be an inadvertent side effect of sampling or husbandry procedures, for instance, by selecting a small segment of a population as breeders to produce the next generation. Selecting individuals with specific characteristics or phenotypes may increase the intensity of selection, and lead to loss of genetic variance. For example, in captive colonies of the Mallorcan midwife toad *Alytes muletensis* maintained as breeding stock for reintroductions, allelic richness and heterozygosity both declined in long-term captive bred stocks compared to short-term stocks and wild populations (Kraaijeveld-Smit *et al.*, 2006). The consequences of selection may be a depression in fitness-related traits (e.g., fertility, disease resistance, growth rate) such as those that are related to survival and reproductive success. Consequences of selection in captive breeding programs are most important in situations where captive-reared individuals are released back into their native environment

or when there is the possibility of breeding with wild individuals. Genetic monitoring of captive breeding and reintroduction programs is important to ensure that artificial selection does not impede continued success. For turtles and tortoises, there is currently little or no genetic monitoring of successful captive breeding and reintroduction programs (Ballou and Lacy, 1995; see also Syed *et al.*, 2007).

Humans exert an ever-increasing influence on the direction and force of selection acting on species. Average global atmospheric temperatures have increased by approximately 0.6^{o}C from pre-industrial times to the year 2000, a rate of change much larger than that seen in the past ten thousand years (Houghton, 2005). By the year 2100, average global atmospheric temperatures are projected to rise by 2^{o}C to 6^{o}C (Mann and Jones, 2003). To put this predicted shift into perspective, this degree of climate change is one third of that seen in the last ice age that lasted a period of approximately one hundred thousand years (Houghton, 2005). Such dramatic climatic changes will exert strong selective pressure on species to evolve. For instance, even moderate temperature shifts (i.e., as little as 2^{o}C for the painted turtle, *Chrysemys picta*) can drastically skew sex ratios in reptiles with temperature-dependent sex determination (Janzen, 1994). Skewed sex ratios can result in smaller **effective population sizes**, elevating risks of inbreeding and loss of diversity via drift. Behavioural modifications, such as nest-site choice and altered timing of the initiation of nesting, may compensate for the effects of these local climatic shifts on sex determination (Doody *et al.*, 2006), although selection would also act on other aspects. For example, juvenile mortality may increase as turtles experience prolonged higher temperatures; reduced hatchling recruitment was found in *Chrysemys picta* after a particularly long hot summer in 1988 (Janzen, 1994). Given these startling projections, can turtles and tortoises evolve at a pace that is rapid enough to compensate for the negative fitness consequences of global warming?

Theory predicts that the maximum rate of sustainable evolution for a population, or conversely, the maximum rate of environmental change that can be tolerated, can be inferred on the basis of the interactions of evolutionary forces on quantitative genetic variation (Lynch and Walsh, 1998). In the absence of immigration, the rate of phenotypic evolution can become limited by the availability of **additive genetic variance**. If the rate of environmental change is too high, selective pressures (e.g., impacting survival and/or

fecundity) could exceed a population's capacity to assimilate new genetic variation via mutation and maintain a positive growth rate, especially for organisms with long generation times such as turtles. If so, the inevitable outcome would be extinction. If the rate of environmental change is sufficiently slow, and if the amount of genetic variation relative to environmental variation is sufficiently high, the population may be able to evolve very rapidly in response to this change. Overall, the capabilities of turtles to respond to and survive the impacts of environmental change such as global warming will depend on the rate of climatic change (i.e., the intensity of selection) and the degree of genetic variance within each population for the key traits. In the face of global warming, maximizing the adaptive genetic diversity at the population, landscape, regional, and species scales is paramount to the survival of turtles and tortoises in the 21st century and beyond.

5) Gene flow and management units

Gene flow is defined as the movement of alleles from one population to another. Such migration is an evolutionary force that counters the effects of genetic drift and inbreeding within each population. Gene flow among populations is often summarized as the average fraction of individuals in each population in each generation that has contributed genes derived from another. Gene Flow can be measured directly from field techniques of mark-recapture and tracking individuals, and indirectly by applying various mathematical models of population structure to genetic data (i.e., the island model vs stepping stone model vs isolation-by-distance model).

There are several reasons to expect that direct measures of movements may differ from indirect measures of gene flow (Slatkin, 1985). First, gene flow in the strict sense refers to the transfer of genes from one population to another. Migration, as quantified by direct observations, documents the physical presence of an individual in more than one population at two or more time periods. Direct observations provide no information about the likelihood of breeding, and thus actual gene flow per se. Further, inferences from direct observations are only germane to those populations where observations were made. Gene flow can occur over much broader areas and the indirect genetic-based estimates can provide accurate measures from population to landscape scales.

Further, direct observations chronicle the extent of movements only over the period of observation but provide no information regarding historical levels of dispersal. Genetic

measures of gene flow report the cumulative effects of past and contemporary gene flow. However, for many populations of conservation or management concern, present levels of gene flow are of special interest. If rates of gene flow and/or effective population size had historically been high, then estimates of gene flow may not reflect present conditions. For example, high levels of gene flow and little population genetic structuring (panmixis) were documented for the geometric tortoise (*Psammobates geometricus*). Populations of *P. geometricus* are severely fragmented, and the indirect measures of gene flow reflect the historical high levels of connectivity rather than the current fragmented condition. In contrast, direct and indirect methods for estimating gene flow yielded similar results in the freshwater turtle *Hydromedusa maximiliani*, with very restricted movements suggesting a **metapopulation** structure within drainages (Souza *et al.*, 2002).

Understanding the use of terrestrial and aquatic habitats by local breeding populations of amphibians and reptiles is critical for conservation and management (Semlitsch and Bodie, 2003). Freshwater turtles often require different habitats to carry out all life-history functions. Turtles often live and forage in temporary wetlands that are some distance from permanent wetlands. They use upland habitats to disperse seasonally between wintering, breeding, and foraging sites, for purposes of aestivation, feeding and hibernation, and females use upland habitats to nest (Burke and Gibbons, 1995). For example, high levels of gene flow in the estuarine diamondback terrapin (*Malaclemys terrapin*) within estuaries are most likely promoted by mating aggregations during the breeding season and high juvenile dispersal (Hauswaldt and Glenn, 2005). These movements were not detected in long-term mark recapture studies (Gibbons *et al.*, 2001) and may be important for inbreeding avoidance and maximizing genetic diversity in estuaries.

Landscape connectivity, the degree to which landscape features facilitate or impede movements and gene flow between populations (Taylor *et al.*, 1993), is an essential feature of landscape structure because of effects on movements among populations, population persistence, and probabilities of recolonization. Landscape connectivity can be quantified in a relative sense based on indices that characterize the spatial dispersion of landscape habitat types and account for the proportional contributions of each landscape type to landscape matrices between populations. The degree of genetic differentiation among populations has been widely used in wildlife studies as a surrogate measure of dispersal (Scribner *et al.*, 2005). For example, Scribner *et al.* (1986) used protein **allozymes** to

estimate genetic relationships among populations of slider turtles (*Trachemys scripta*) that were separated by different types of intervening habitats. Based on estimates of inter-population variance in **allele frequency**, these authors presented compelling evidence for higher rates of gene flow among populations from different embayments along contiguous lake shoreline relative to interspersed (but aquatically connected) riverine habitat. Populations in small ponds separated by upland terrestrial habitat had the lowest rates of gene flow compared to those in the other intervening habitat types.

Management strategies for populations need to account for the dispersal capabilities and natural history of the species. Where panmixis occurs, the populations may be managed as a single entity with a focus on maintenance of size and habitat quality. In contrast, where there is a high degree of structuring, each population contributes to overall species diversity. Managing these populations as separate units is important to ensure diversity is retained within each, and that overall species diversity is not compromised from increased gene flow and resultant genetic homogenization (DeYoung and Honeycutt, 2005; Moritz, 1994; Moritz, 1999). Mixing genetically differentiated populations can also cause outbreeding depression (see section 3). Management can be guided by the extent to which populations have diverged, with issues of outbreeding depression and isolation being of greatest concern among the most divergent units, referred to as "**evolutionary significant units**" (Moritz 1994), in comparison to less divergent populations referred to as "**management units**".

Spinks and Shaffer (2005) defined management units for the vulnerable western pond turtle (*Emys marmorata)* with analyses of 1372bp of *ND4* and *tRNA* mitochondrial genes. Populations in northern California and farther north were genetically similar and formed a single management unit, whereas drainages farther south exhibited more structuring. In central and southern California, a large proportion of intraspecific diversity could be attributed to two populations. To retain diversity, these two populations should be a priority for conservation and management of *E. marmorata*.

Defining management units was a greater challenge for the giant Amazon River turtle, *Podocnemis expansa*. This species has an impressive dispersal capability, with females known to traverse up to 400km between nesting beaches and feeding areas (Von

Hildebrand *et al.*, 1988). As predicted from theory, because of its dispersal capabilities and lack of barriers to dispersal, high levels of gene flow were found within basins (Pearse *et al.*, (2006a). Based on this mtDNA analysis, an entire basin represents a management unit. Lack of structuring in basins was confirmed for nine microsatellite loci but these markers also revealed recent reductions in population size. Extensive harvesting has decimated populations of *P. expansa* and its continuation will result in loss of genetic diversity. Given the harvesting pressures, the units of management would be more appropriate at the population level to ensure local nesting beaches are not overexploited for eggs and mature females of *P. expansa.* Conservation biologists thus need to consider all threatening aspects from local to landscape scales when defining units for management in chelonians.

6) Clarifying taxonomy

Inadequately informed management plans and a limited knowledge of biological richness are often the result of misunderstanding taxonomic status and relationships among taxa. If the units of evolutionary significance or taxonomic importance have not been identified and prioritized for conservation, biological diversity may not be protected adequately. Molecular methods are particularly amenable to resolving taxonomic relationships and identifying units for conservation, because they can uncover diversity in taxa not apparent from morphological analyses. Phylogenetics is a discipline that often uses genetic information to delimit species boundaries and divergent lineages within species, and then to estimate the evolutionary relationships amongst those units (Avise and Wollenberg, 1997; Davis and Nixon, 1992; Nei and Kumar, 2000; Iverson *et al.*, 2007; Bickham *et al.*, 2007). We will illustrate how phylogenetics has contributed to resolving taxonomic issues in chelonians.

Taxonomy has traditionally used morphological characters to delimit species where a **holotype** is used as a reference specimen. However, the propensity of some turtles to hybridize with other species can result in difficulties. For example, at least two "species" of rare Chinese turtles were described from specimens purchased from the Hong Kong animal trade. Scientists were unable to find these animals in the wild and began to question their taxonomic validity. Allozyme and **mitochondrial** DNA analyses revealed that these "taxa" were not representative of species but rather they were distinct morphological forms resulting from **hybridization** events (Parham *et al.*, 2001). Hybridization and **introgression** are fairly common in freshwater turtles (e.g., (Spinks and Shaffer, 2005;

Stuart and Parham, 2004; Georges *et al.*, 2002). Neutral genetic markers may effectively resolve these taxonomic issues and have advantages over morphological traits as they are less subject to plasticity and presumably selection.

Phylogenetic studies can redefine taxonomies. Taxonomies have been refuted or supported by genetic evidence where phylogenetic criteria are used to delimit species and genera (reviewed in Bickham *et al.*, 2007). Delimiting species on the basis of combined molecular and morphological criteria is considered the best approach for resolution of taxonomies (Dayrat, 2005; Blaxter, 2004; Seberg *et al.*, 2003). For turtles and tortoises, delimiting species boundaries can be even more difficult because interspecific hybridization frequently occurs even amongst distantly related taxa (e.g. Georges *et al.*, 2002). Phylogenetic methods can identify such instances of hybridization and resolve taxonomies to define groups constituting genera or species (Sites and Marshall, 2004; Templeton, 2001). For example, in a phylogenetic study of the Geoemydidae, not all recognized species appeared to be of the same evolutionary lineage. This suggested misclassification of several species (by some criteria), and instances of interspecific hybridization were documented. Based on this genetic evidence, taxonomic revision of this group was required (Spinks *et al.*, 2004).

Phylogenetic or phylogeographic studies can identify cryptic species. Cryptic species are named because they comprise distinct genealogical lineages but in the absence of molecular or behavioural evidence, lack distinguishing morphologic characteristics or other diagnostic features to warrant recognition as species. For purposes of conservation, cryptic species are important units of diversity and may represent threatened taxa, previously unknown to conservation biologists (Fritz *et al.*, 2005; Walker *et al.*, 1998; Georges and Adams, 1996; Georges *et al.*, 1998). In Asian softshell turtles, two species were formally recognised in the *Chitra* genus: *C. indica* and *C. chitra*. Mitochondrial DNA sequence data revealed three deeply divergent **monophyletic groups** in *Chitra* (Engstrom et a., 2002). The third and previously unidentified form was subsequently named as a distinct species (*C. vandijki*) based on additional morphological data (McCord and Pritchard, 2002), and is a critically endangered species that warrants greater protection (Engstrom *et al.*, 2002). As protection is often conferred to recognized ‘species’ or ‘subspecies’ in wildlife legislation, it is imperative that taxonomies are clearly defined for

effective conservation (Soltis and Gitzendanner, 1999; George and Mayden, 2005; Bickham *et al.*, 2007).

7) Insights into species biology

Biologists have traditionally explored various aspects of the natural history of a species through observation. Turtles are notoriously difficult subjects for some observational studies, yet knowledge of many aspects of a species' biology is critical for successful conservation efforts. **Molecular markers** are providing new insights into turtle mating systems, dispersal (sex-specific or otherwise), population connectivity, and fluctuations of population sizes that can be difficult to ascertain from field and observation studies alone.

Female turtles have sperm storage structures in the oviducts (Gist and Jones, 1989), and captive females held in the absence of adult males were known to produce viable eggs for as long as seven years (Ewing, 1943; Magnusson, 1979). Molecular marker studies have revealed that freshwater turtles and tortoises in natural populations frequently use stored sperm to fertilize eggs (e.g. Gist and Congdon, 1998; Pearse and Avise, 2001; Roques *et al.*, 2004). Indeed, microsatellite DNA analyses have revealed that some *Chrysemys picta* will produce fully-fertile clutches of eggs in nature without re-mating for three years (Pearse *et al.*, 2002). However, lower hatching success and hatchling mass were found in clutches fertilized from stored sperm in the European pond turtle (*Emys orbicularis*), suggesting deterioration of stored sperm for some species (Roques *et al.*, 2006).

The vast body of literature documents a substantial frequency of multiple paternity in non-marine turtles and tortoises (Moon *et al.*, 2006; Palmer *et al.*, 1998; examples include Galbraith, 1993), but there are exceptions. Low incidences of multiple paternity (less than 10% of clutches) have been documented for *Emys orbicularis*, resulting perhaps from competition of viable stored sperm to fertilize eggs (Roques *et al.*, 2006). This finding contradicted observations of multiple *E. orbicularis* males mounting a single female during the breeding season (Rovero *et al.*, 1999). Mating systems may also differ between populations of the same species. *Podocnemis expansa* exhibits 100% multiple paternity in some populations (Valenzuela, 2000) and only 10 to 20% in others (Pearse *et al.*, 2006b). Molecular markers thus can shed light on mating systems in turtles and tortoises that may not be apparent from observation data.

Reproductive success is critical to population persistence. Only recently, based on applications of biochemical markers have turtle biologists been able to extend estimates of annual recruitment to quantify reproductive contributions of individual adult males and females. Variance in reproductive success will greatly affect Ne and generational rates of loss of genetic diversity. Importantly, knowledge of phenotypic, demographic, and geographical (e.g., habitat) variables that can be linked to reproductive success and to inter-annual variation in recruitment will greatly aid in the development of conservation plans. Scribner *et al.* (1993) used allozymes to examine relationships between inter-annual variation in reproductive success and juvenile cohort measures of genetic diversity *Chrysemys picta* that inhabits the E.S. George Reserve, a large protected wetland complex in southeastern Michigan, During years where few females successfully reproduced, offspring from these cohorts were characterized by higher **inbreeding coefficients (F)**, lower heterozygosity (H), and higher genetic correlations among individuals (θ) compared to cohorts recruited in years when greater proportions of females contributed progeny. For conservation biologists, these findings emphasize that factors affecting inter-annual variation in recruitment also can impact cohort levels of genetic diversity.

Ecological characteristics are not alone predictive of how genetic variation is apportioned within and among populations. Closely related turtle species may display substantial variation in connectivity and structure that reflect important differences in natural history among species. For example, Roman *et al.* (1999) found strong phylogenetic structuring for the highly aquatic alligator snapping turtle (*Macrochelys temminckii*) across basins in a mtDNA control region analysis, suggesting limited dispersal of turtles. In contrast, *Chelydra serpentina* lacked structure for allozyme and mtDNA, reflecting its perhaps greater ability to disperse over land and long distances in water (Phillips *et al.*, 1996). Each species is different. The most informed conservation decisions are formulated based on knowledge of fundamental aspects of a species' biology derived from joint studies of genetic structure and natural history.

Estimating the size of a population from mark-recapture analyses can be difficult and time-consuming, particularly for species that are difficult to capture or at low population densities. Obtaining genetic samples can be easier because individuals do not need to be subsequently re-caught to obtain data for estimating population size. Molecular data can be used to estimate the effective population size, which is the size of the population that is

actually reproducing, a parameter that may be more meaningful for conservation than the census size. The effective population size (Ne) can be monitored by assessing temporal changes of allele frequencies in the population (Luikart *et al.*, 1999; Richards and Leberg, 1995). Genetic techniques can also provide point estimates of the number of breeding individuals in a population (Nb) from paternity (or maternity) microsatellite data. Pearse *et al.* (2001) developed a technique for estimating current reproductive size of a population of *Chrysemys picta* and provided additional information, such as the movement of breeding individuals, which was not possible based on capture-mark-recapture studies alone.

8) Forensics

Trade in turtles has increased dramatically and is considered to be the greatest threat to their survival (Asian Turtle Working Group, 1999). Turtle and tortoise trade can be classified into three main categories: trade for human consumption, pet shop trade, and traditional medicines (Turtle Conservation Fund., 2002). Consumption of turtles is by far the largest scale trade, and larger, more mature individuals tend to be targeted (Asian Turtle Working Group, 1999). Due to their life-history characteristics (great longevity, high juvenile mortality, and late onset of maturity), this type of trade probably has the greatest negative impact on chelonian populations (Asian Turtle Working Group, 1999; Smith, 1993; van Dijk, 2000). Exploitation of chelonians for the pet shop trade favors juveniles of unusual species and, as commodity values are often driven by rarity, this can rapidly contribute to the extinction of rare and endangered species (Ceballos and Fitzgerald, 2004; Cheung and Dudgeon, 2006; Gamble and Simons, 2004; Gong *et al.*, 2006). Finally, large numbers of turtles are frequently harvested primarily for their shells, which are ground to a powder or jelly, and sold for its alleged positive effects on longevity and virility in humans (Hsieh *et al.*, 2006; Van Dijk *et al.*, 2000; Lo *et al.*, 2006).

DNA-based forensic methods can be used to monitor illegal trade by verifying taxonomy and providing information on geographic origin of seizures. Traditionally, morphological characteristics were used for species identification. However, often seizures include small fragments of eggshells, carapace, cooked meat, or powdered turtle shell, where standard diagnostic features are no longer discernible. Molecular methods are ideal for forensics because they can be used on degraded or processed specimens, and can elucidate species, and even regional or population origins (Randi, 2003). Where commercial industries are established, genetic techniques may be the only means by which products derived from

legal trade can be reliably distinguished from poaching activities. Further, genetic methods have the resolution to 'tag' individuals and establish paternities or maternities, technologies that are particularly useful for monitoring activities of licensed reptile breeders. The application of molecular techniques for wildlife forensics is still in its infancy. Approaches tend to be handled on a case-by-case basis and standard protocols have not been adopted. Currently only a few studies have applied molecular techniques for forensic issues in freshwater turtles and tortoises.

Legitimacy of turtle meat trade in Florida and Louisiana were investigated by Roman and Bowen (2000). Species composition was determined from 36 turtle meat products purported only to contain *Macrochelys*. The majority did not contain *Macrochelys,* but were predominantly *Chelydra serpentina*, as revealed by analyses of the control region and cytochrome *b* genes of mtDNA (394bp and 256bp respectively). This shift in trade to a species that is 50kg lighter in weight and less favored for its flavor is speculated to reflect depletions of *M. temminckii* populations. With more catch effort required by harvesters to meet demand from these depleted populations, the market shifted to the more readily available *Chelydra*. In addition, softshell turtles (*Apalone* spp.) were present in a small proportion of the products. Impacts of this trade have not been investigated for any of these species, although current harvest rates may not be sustainable. Further research on the effects of harvesting and continued genetic monitoring of processed trade goods is recommended to prevent overexploitation or to minimize its impact in these species.

Molecular methodologies have analysed species composition in cooked meat, eggs (Moore *et al.*, 2003), and powdered turtle shell (Lo *et al.*, 2006). Preparations of turtle shell in the Taiwanese market were analysed with mitochondrial 12s ribosomal RNA and cytochrome *b* sequences (Lo *et al.*, 2006). Reassuringly, CITES (Convention on International Trade of Endangered Species of Wild Fauna and Flora) listed species were not present in turtle shell and jelly preparations. Also in Taiwan, methods have been developed for determining the presence of a CITES listed endangered turtle *Kachuga tecta,* in shell preparations (Hsieh *et al.*, 2006).

Identifying geographic origins or provenance of seizures is required to repatriate animals to their wild populations without disrupting existing genetic structure or elevating risks of outbreeding depression. Molecular techniques can also be used for assessing origins of

individuals. In the case of the Indian Star Tortoise (*Geochelone elegans*), the origins of 92 individuals seized from the Singapore airport were determined using mtDNA (control region, cytochrome *b*) and six microsatellites (Gaur *et al.*, 2006). The rescued group of tortoises was found to be a mix of individuals from different populations in southern India and possibly Sri Lanka. Exact localities for many of the individuals could not be identified because sampling was limited and not all diversity had been characterized across the range of *G. elegans*. With more extensive sampling, these methodologies will be able to identify source populations of seized chelonians, enabling them to be returned to their original geographic location(s). Overall, these studies highlight the power of molecular methods to monitor trade directly from a range of trade products for species identification and provenance delineation.

The utility of genetics in forensics is hindered by the limited markers available for chelonians. With more markers becoming available from genome sequencing projects, such as that proposed for *Chrysemys picta* (refer to http://www.reptilegenome.com for more information), genetics will play an ever-increasing role. New technologies, such as single nucleotide polymorphisms (SNP) markers will enable analyses of samples from more highly degraded samples, more rapidly and with greater resolution for addressing forensic issues. Advances in genetic technologies and marker development will pave the way for development of DNA registers for routine monitoring of trade activities. Such inventories are urgently required if we are to assess the threats of overexploitation to turtles and tortoises worldwide.

Concluding remarks

We have discussed important genetic issues that conservation biologists should consider when planning and executing projects involving turtles. We have highlighted the importance of genetic diversity for future adaptive evolution and we outlined processes by which diversity is lost. Anthropogenic effects can exacerbate loss of genetic diversity owing to increased habitat fragmentation and diminished population size. Genetic approaches can be used to detect and monitor these effects at various temporal and spatial scales.

Understanding historical and contemporary evolutionary processes, at scales ranging from an individual to an entire landscape, provides valuable knowledge for development of

short-term and long-term conservation plans. Conservation priorities can be identified and program success can be monitored using molecular methodologies. Aspects of turtle biology and mating systems that are exceedingly difficult or impossible to ascertain from field studies can be illuminated using genetic markers. Further, molecular methods are an emerging crime investigation tool for monitoring the turtle trade. Despite these applications and the inherent importance of genetic diversity to long-term viability of turtle populations, there is a general paucity of such genetic studies on freshwater turtles and tortoises (reviewed in FitzSimmons and Hart, 2007)

Due to the lack of studies, there is a limited repertoire of molecular markers currently available for turtle geneticists (Engstrom *et al.*, 2007). With the ongoing genomic revolution, the number of available markers, their information content, and range of applications for chelonian conservation will greatly increase. For example, new genomic approaches offer exciting possibilities to investigate whether variation within specific gene regions can be tied to phenotypic or other traits that are tied to probabilities of survival or reproductive success. Emerging technologies hold great promise to link increasingly assessable modern technology to fundamental problems in turtle biology and conservation. Other technological advancements will enhance efficiency of DNA fingerprinting technologies and enable high throughput analyses, such as SNPs (**single nucleotide polymorphisms**) and **microarrays** (reviewed in McGaugh *et al.*, 2007).

We conclude by listing what we perceive to be three crucial future directions in turtle conservation genetics:

1. Reconciling taxonomic uncertainties and identification of genetic discontinuities at landscape and species levels to delineage management units.
2. Predicting effects of landscape-level changes and concomitant changes in population demography and movement patterns on apportionment of genetic diversity within and among populations.
3. Monitoring trade and directing enforcement to protect overexploited turtle populations.

Each issue is a global concern that potentially influences every turtle species. While substantial progress has been made, the geographic and taxonomic coverage has been uneven and not necessarily focused on species of greatest concern (reviewed in

FitzSimmons and Hart, 2007). Turtle geneticists should work closely with biologists, managers, local communities, and conservation organizations to bring state-of-the-art technology and methods of statistical inference to bear on pressing issues in turtle conservation.

Glossary of genetic terms

Additive Genetic Variance- Genetic variance that arises from the additive effects of genes on the phenotype.

Allele- Alternative forms of a gene at a given locus on a chromosome.

Allele Frequency- Also termed gene frequency. The proportion of an allele (or gene) in a population relative to other alleles (or genes) at its locus.

Allelic Richness- The number of alleles in a population corrected for sample size. Used as a measure of genetic diversity.

Allozymes - Forms of an enzyme that differ in amino and have different electrophoretic mobilities.

Chromosome- A strand of DNA with associated proteins that is visible as a rod-shaped structure in cells that have been stained during cell division. Chromosomes contain the heritable genetic information within the DNA.

Deleterious Recessive Alleles - The phenotypic effects of recessive alleles are masked in the phenotype of heterozygotes, and expressed in homozygotes. Deleterious alleles have negative fitness effects on individuals.

Effective Population Size- The average number of breeding individuals in a population which are assumed to contribute equally to the next generation.

Evolutionary Significant Unit - A population (or group of populations) reproductively isolated from other conspecific population units for long enough duration to display genetic isolation, and is an important component in the evolutionary legacy of the species.

Epistasis- The interaction between two nonallelic genes, such that one gene interferes with the expression of the other at a different locus.

Fitness- The ability of an individual to produce offspring in a given environment. In a genetic sense; the relative reproductive success of a genotype.

Founder Effects- The loss of genetic diversity when a new colony is formed by a very small number of individuals from a larger population; a form of genetic drift.

Gene- A basic unit of inheritance transmitted through the gametes from generation to generation, occupying a specific locus on a chromosome and with a specific function.

Gene Pool- All the genes available among reproductive members of a population at a given point in time.

Genotype- The genetic constitution of an individual.

Genome The entire complement of genetic material in a cell. In eukaryotes this refers to the genetic material in a single set of chromosomes.

Genetic Drift- Changes in allele frequencies of populations due to random sampling effects because not all individuals (and their genes) will reproductively contribute to the next generation.

Gene Flow- Movement of genes from one population to another by interbreeding or migration.

Genotypic Frequency- The proportion of a genotype in the population relative to all other genotypes.

Heritability- The proportion of phenotypic variability for a given trait that is genetically based; expressed as the ratio of genetic variance to phenotypic variance.

Heterosis- Superiority or vigour of hybrid individuals compared to either parental stock.

Heterozygote- A diploid individual with different alleles at a particular locus.

Homozygote- A diploid individual with identical alleles at a particular locus.

Holotype- The single specimen designated or indicated as the name-bearing type of a nominal species or subspecies by the original author.

Hybridization- Crossbreeding of individuals of different genetic composition, typically belonging to different species or varieties to produce hybrid offspring.

Inbreeding- Mating of related individuals.

Inbreeding Coefficients (F)- The probability that an individual contains copies of the same ancestral gene from both its parents because they are related.

Inbreeding Depression- Reduction of fitness by increased homozygosity as a result of inbred matings.

Introgression- The spread of genes from one species to another via hybridization and backcrossing.

Locus- The specific region on a chromosome where a gene is located (plural = loci).

Management Units- Demographically independent sets of populations identified to aid short-term conservation management. Genetically divergent but not to the extent as observed in evolutionary significant units.

Meiotic Drive- Preferential production of certain gametes during meiosis (germ cell production). This alters the expected Mendelian segregation ratios in heterozygotes.

Mendelian Segregation- Mendel's first law. The principle that the two different alleles of a gene pair segregate from each other during meiosis; each resultant gamete has an equal probability of obtaining either allele.

Metapopulation- A group of spatially separated populations from the same species connected by immigration and emigration.

Microevolution- Evolutionary events occurring over a shorter period of time, such as the changes in the gene pool of a population.

Microsatellites- Tandem repeat motifs of DNA sequence interspersed throughout the eukaryotic genome in which the repeat unit is typically five or fewer bases in length.

Molecular marker- A genetic polymorphism with multiple alleles and a simple mode of inheritance. Useful in pedigree studies, disease studies, studies of the distribution of genes in populations and linkage mapping.

Mutation- A change in a gene or chromosome.

Microarrays- A technique used to monitor gene expression in which genes or gene fragments are deposited typically on a glass, filter, or silicon wafer in a predetermined spatial order allowing them to be made available as probes.

Migration- Movement of an individual or group from one location to another.

mtDNA- Mitochondrial DNA: The circular, double-stranded DNA of the mitochondria. It typically has matrilineal inheritance, although paternal leakage has been documented for some taxa.

Monophyletic Group- A group comprised of a single ancestral species and all its descendants. Also called a clade.

Natural Selection- A primary mechanism for evolution in which individuals best suited to their environment have greater survival and reproductive success, thereby transmitting their advantageous genetic characteristics to succeeding generations.

Neutral Genetic Markers- Genetic markers presumably not under the forces of natural selection and often residing in non-coding genomic regions.

Outbreeding- The breeding of genetically unrelated or distantly related individuals.

Outbreeding Depression- A reduction in the fitness of progeny from matings of individuals from different populations, possibly from the breakdown of co-adapted gene complexes or ‘swamping’ of locally adaptive genes.

Panmictic- Pertaining to a genetically unstructured randomly mating population.

Phenotype- The observed properties of an organism, resulting from the interaction of its genotype with the environment.

Phenotypic Plasticity- The ability of an organism's phenotype to change in response to changes in the environment.

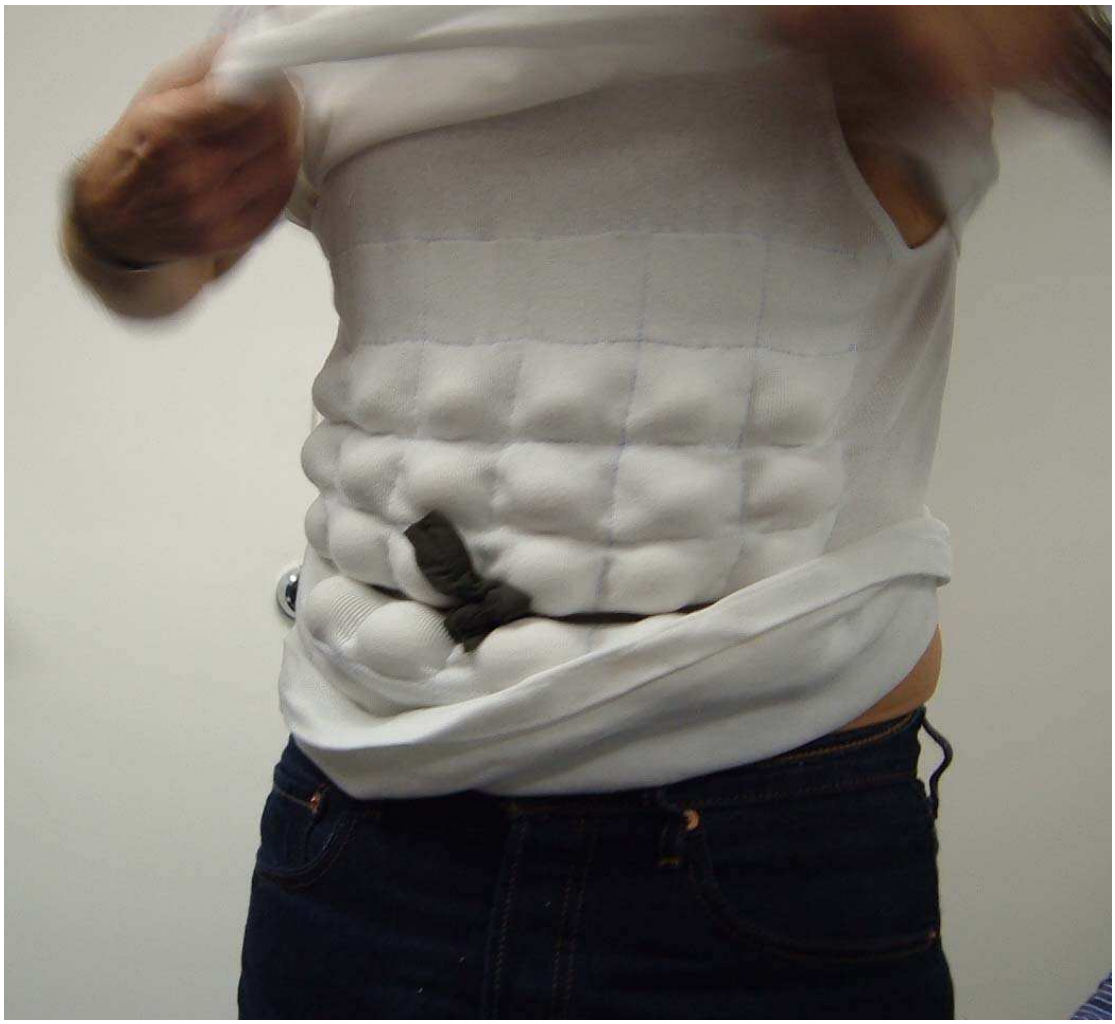
Population Bottleneck- An evolutionary event resulting in a decrease in the size of a population and subsequent loss of genetic diversity via the effects of genetic drift.

Quantitative Genetics- The study of the genetic basis of traits showing continuous variation.

Single Nucleotide Polymorphism- Variations in DNA sequence that occur when a single nucleotide base (adenine, guanidine, cytosine, or thymine) is altered via a mutation event.

Vicariance- The splitting of closely related groups of taxa or biota by the formation of a natural barrier.

Chapter 3 – Wildlife across our borders: a review of the illegal trade in Australia.



Picture: A perpetrator wearing a vest that is custom built for the purpose of smuggling bird eggs past airport security. Photo by Australian Customs Service.

Chapter 3 – Wildlife across our borders: a review of the illegal trade in Australia.

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Abstract

Australian flora and fauna are highly sought for the international black market in wildlife – the second largest illegal global commerce. Within Australia, trade in exotic wildlife supplies avid hobbyists. Using data on wildlife seizures by Australian Customs between 2000 and 2007 and case prosecutions from 1994 to 2007, we assessed the scale and enforcement of wildlife crime in Australia. Most seizures were minor; less than 1% resulting in prosecution of the persons involved. Of cases prosecuted, 46% were for attempted export and 34% for attempted import. Reptiles were targeted most (43%), then birds (26%), and native plants (11%). 70% of prosecutions was a fine only (maximum of $30,000) consistently less than the black market value of the seized goods. Prison sentences increased from an average of 10 months (between 1994 and 2003) to 28 months (between 2004 and 2007). Formation of the Australian Wildlife Forensics Network and ongoing support from the Australian Federal Police for research into improved options for policing are exciting developments. Priority for effective regulation of legitimate commercial trade and effective policing of illegal trade is likely to increase in coming years as trends toward greater globalization of commerce continue and restrictions on trade relax.

Introduction

Australia has such a rich and unique biota that it has gained international recognition as one of the 25 biodiversity hotspots, particularly in the south west of Western Australia (Myers, Mittermeier *et al.* 2000). These hotspot regions have been identified based on the large number of species present and the high proportion of species that are found nowhere else in the world (i.e. endemic species). Over 80% of Australian flora and fauna are endemic and it is this very attribute that attracts traders of illegal wildlife worldwide. Illegal trade of wildlife is a serious and growing crime and worth more than US $20 billion dollars per year (Interpol 2007). Highly organised criminal networks spanning several

countries including Australia have been implicated in large scale wildlife smuggling operations. Such operations are often not only cruel, with many animals dying in the process, but also endanger wild populations because overexploitation to supply the illicit trade can rapidly cause extinction. Exotic species that are smuggled into Australia also pose a significant biosecurity risk because they can potentially establish themselves in the wild and become pests. They can also carry seeds, parasites, and viruses which, if released, to the environment would have devastating impacts on native fauna and flora, and on the agricultural and aquaculture industries.

Despite the black market commerce in wildlife being of considerable risk to Australia's unique biodiversity and to our industries, there are no recent studies describing the extent of the illegal trade in Australia. Using data from the Australian Customs Service of wildlife prosecutions over the past 13 years, this review describes the extent of illegal trade of wildlife in Australia with a focus on wildlife that is imported into or exported out of Australia. Case prosecutions for illegal trade of wildlife and wildlife products in Australia are typically dealt with by Magistrates Courts and are not reported, so they do not reside on any standard legal databases. Instead, we drew data from the wildlife prosecutions database of Australian Customs Service for the period of 1994 to 2007, and examined the records to assess whether illegal trade operations are on the increase, which taxa are targeted, and the types of penalties they incur. We also describe new technologies, such as DNA technologies, that can be used to provide evidence for prosecutions of illegal trade of wildlife. Finally we suggest future directions of services to detect and provide evidence for wildlife crime in Australia.

Australian wildlife legislation

The Convention on International Trade of Endangered Wild Fauna and Flora (CITES), to which Australia is one of 172 signatories, was established in 1963 and aims to ensure that international trade in wild specimens of plants and animals does not threaten their survival. The CITES agreement provides a framework for signatories to adhere to and to enforce the treaty via their domestic legislation. Animals and plants are listed in three appendices accordingly to their vulnerability of extinction from overexploitation for trade. CITES Appendix I listed species include those threatened with extinction that can not be traded except under exceptional circumstances. Appendix II species require trade controls to

ensure their survival and Appendix III species are protected in at least one country with assistance required from other signatories to control their trade.

In Australia, adherence to CITES is regulated under Part 13A of the Commonwealth *Environmental Protection and Biodiversity Conservation Act* 1999 (EPBC Act). The Act regulates the export of Australian native species (unless identified as exempt), exports and imports of all CITES listed species, and the import of live plants and animals that could adversely affect native species or their habitats. Penalties for breaches of the EPBC Act (1999) are fines up to AUS $110,000 for an individual or $550,000 for a corporation, and up to ten years imprisonment. In addition, persons may be convicted under State wildlife protection and animal welfare legislation.

Penalties in Australia are more severe than the in the US where the maximum penalty is US$100,000 for an individual or US$200,000 for an organisation and up to one year imprisonment for breaches of *The Endangered Species Act (1973).* Sentences are also more severe than the UK which has a maximum of seven years imprisonment and unlimited fines for breaches of the *Customs and Excise Management Act (1979)* and the *Control of Trade in Endangered Species (Enforcement) (Amendment) Regulations 2005* (COTES).

Legal wildlife trade across our borders

Not all international trade of wildlife in Australia is illegal. Large-scale commercial operations have been established for import and export of wildlife and wildlife products internationally, though the export of live wildlife is effectively prohibited. All commercial operations must be approved by the Australian Government, specifically the Department of Environment, Water, Heritage, and the Arts (DEWHA). Prior to approval a comprehensive wildlife trade management plan meeting the requirements of the EPBC Act (1999) must be submitted; approvals may be granted for up to five years. To export wildlife specimens a permit must be issued by DEWHA. The permit will only be issued if the specimen is sourced from an approved captive breeding, aquaculture, artificial propagation, or wildlife trade operation. Australia's primary commercial wildlife exports are from commercial fisheries, crocodile farms, native flora, and kangaroo meat from approved harvesting operations. A permit is also required to commercially import wildlife into Australia. Species that are CITES II listed and are ranched or harvested from the wild must be sourced from an approved commercial import program. However, amendments to the

EPBC Act made in 2006 (*Environment Protection and Biodiversity Conservation Act (amended) 2006)*, specify that this condition is only required if the species appears on the declared specimens list. This list is subject to change at any time as deemed appropriate by the Minister for Environment. In addition, permits are also issued for imports and exports of wildlife for non-commercial purposes such as for research, education, exhibition, household pets and for personal use.

Illegal wildlife trade across our borders.

Attempted exports and imports of wildlife are usually detected by the Australian Customs Service at airports, in the mail system or through raids on properties as a culmination of investigations carried out by DEWHA or State wildlife enforcement authorities. The majority of wildlife specimens detected that are destined for sale in the black market internationally or within Australia are seized by the Australian Customs Service. Customs is the principal agency managing and securing the integrity of Australian borders. They work in close alliance with the Australian Federal Police, the Australian Quarantine and Inspection Service (AQIS), the Department of Immigration and Citizenship and the Department of Defence.

The total number of wildlife detections and seizures has increased considerably in the past three years, with 7533 seizures in 2006 to 2007, compared to 3902 in 2004 to 2005 (Figure 3.1). The majority of seizures of prohibited wildlife imports involve minor breaches and mostly concern processed wildlife products purchased in international markets. Persons involved in minor seizures are typically not aware that the products they are attempting to bring into the country are prohibited. Major seizures are those in which the persons involved are interviewed or where prosecutions are commenced. The proportion of seizures considered to be major has remained at less than 1% of total detections (Figure 3.2) although there were considerably more major seizures in 2001 and 2002. The increase in the total number of detections since 2004 may be due to increased baggage screening at Australian airports.

The number of prosecutions for illegal trade of wildlife varied from year to year, ranging from 6 cases in 2005 to 14 cases in 2002 (Figure 3.3). The majority of prosecutions from 1994 to 2007 were for attempting to illegally export native flora and fauna (46%).

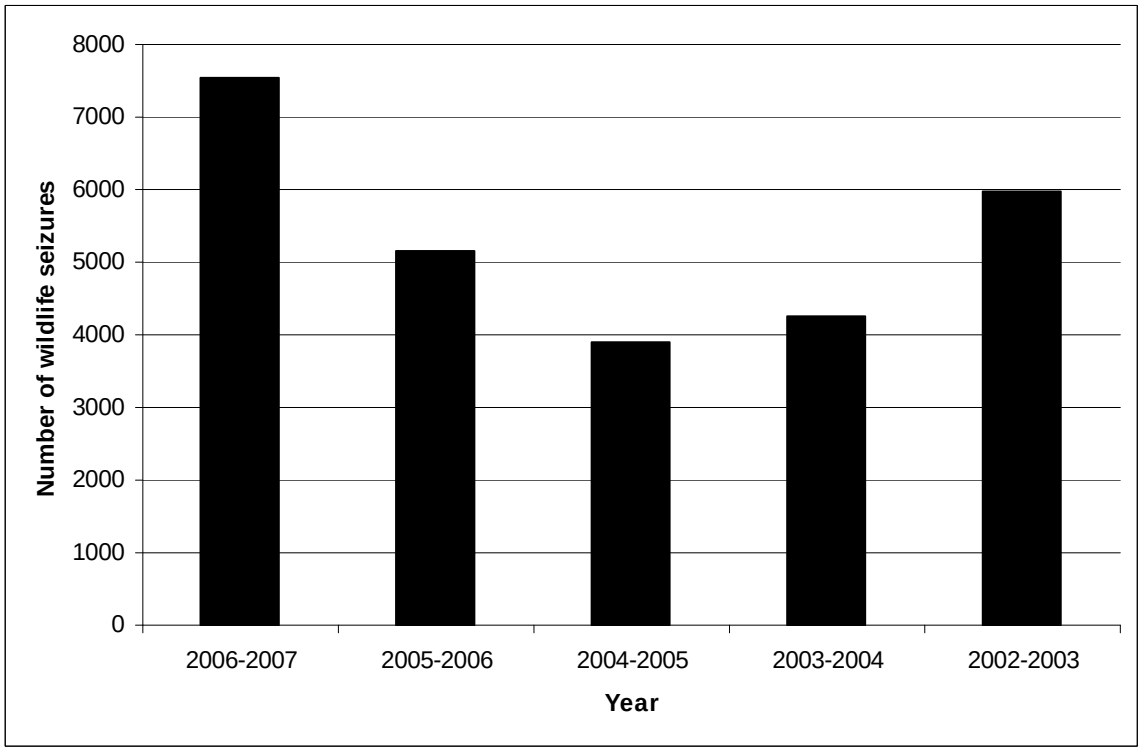


Figure 3.1 The total number of wildlife seizures reported in annual reports of the Department of Environment, Water, Heritage and the Arts from 2002 to 2007 (DEWHA 2002 – 2007). Reported wildlife seizures include attempted imports and exports of plants, animals and derived products.

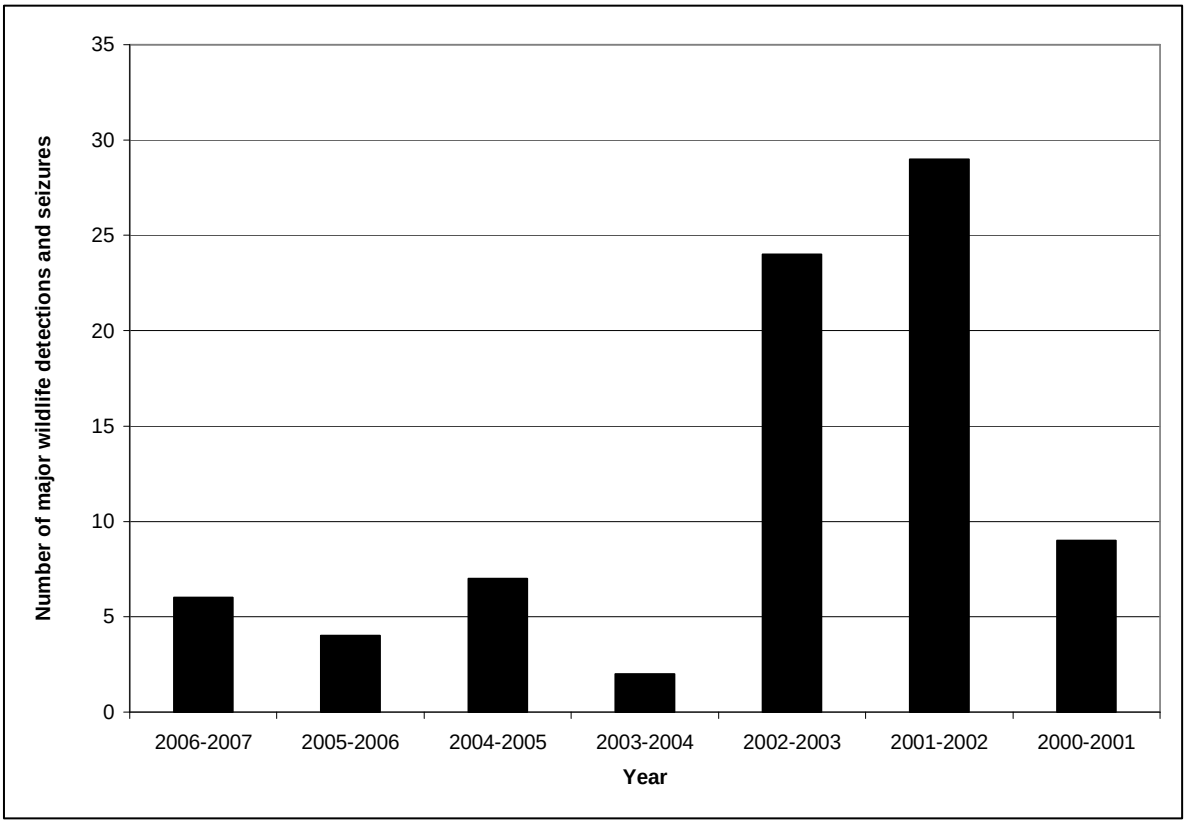


Figure 3.2 The number of major wildlife detections and seizures included attempted imports and exports reported in annual reports of the Australian Customs Service for 2000 to 2007 inclusive (Customs 2000 – 2007). A major find refers to an incident in which the persons involved are interviewed or prosecutions are commenced.

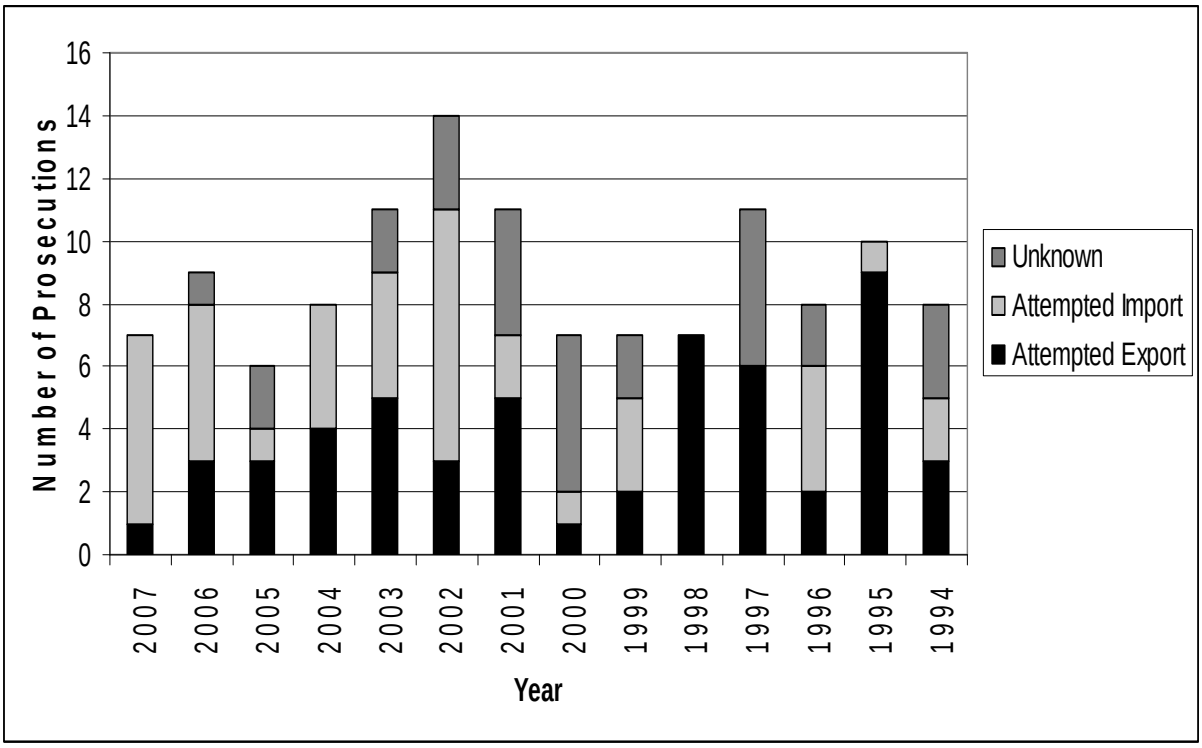


Figure 3.3 The number of prosecutions involving illegal importation and exportation of wildlife in Australia as reported by the Australian Customs Service in annual reports from 1994 to 2007 (Customs 1994 – 2007). Unknown refers to cases in which bird eggs were seized and it could not be established whether they were from native or exotic bird species.

Attempting to illegally import exotic fauna, flora, and wildlife products contributed to 34% of prosecutions, while for the remaining 20% of cases there was no information available as to whether the specimens seized were native or exotic species (Australian Customs Service 2008c). These 'unknown' cases were predominantly seizures of bird eggs where species can be very difficult to identify.

Organised Crime and Wildlife Trade in Australia.

Organised criminal networks conducting large scale illegal wildlife smuggling operations spanning several countries have been detected in Australia. In the most recent case of June 2006, a package containing 25 kg of a powder derived from CITES II listed seahorses was seized in New Zealand. Investigations by Australian Customs Service and the New Zealand Wildlife Enforcement Group revealed that the seahorse powder had been illegally imported into Australia from China, and then illegally exported to New Zealand for sale in conventional medicine outlets (Australian Customs Service 2006a). In September 2004 an international wildlife smuggling syndicate dealing in reptiles and birds from Australia, South Africa and South East Asia was disrupted when raids were conducted on five rural properties in Queensland, Western Australia, New South Wales and Victoria. The raids followed a seizure of 19 pythons and 52 bird eggs by Customs officers at Brisbane airport (Australian Customs Service 2004b). In another case in 2001, six people in the United States, including an Australian connection, were convicted for a million dollar cycad and orchid smuggling operation spanning the United States, Australia, South Africa and Zimbabwe (Australian Customs Service 2007d). The covert and sophisticated nature of these operations spanning several countries makes these criminal networks difficult to detect by local authorities.

The internet and the illegal wildlife trade.

The internet is a convenient medium for illegal wildlife traders to advertise and sell their wares anonymously, and enables direct sales to the buyer thereby eliminating the 'middleman'. In a single week, over 9,000 general listings of animals and animal product were found on the Internet by the International Fund for Animal Welfare (IFAW 2005). For elephant ivory alone, there were 197 listings on eBay Australia of which only two were found to be compliant with wildlife legislation (IFAW 2007). In excess of 2,500 mail packages have been seized at Australia Post Services in Sydney containing commercial weight loss products derived from the CITES II listed Hoodia plant that had been sold over

the internet (Australian Customs Service 2008a). In another case in August 2004, a Western Australian person was convicted for attempting to sell, via the internet, endangered CITES II listed Indian star tortoises, *Geochelone elegans*, which had been illegally imported into Australia (Australian Customs Service 2007b). The internet has become the medium of choice for illegal wildlife traders and regular surveillance of popular internet sites is critical in efforts to curb the illegal commerce of wildlife internationally, and within Australia. However, to our knowledge, Australia does not engage in routine surveillance of the internet to detect wildlife crime. Surveillance is typically undertaken only in support of specific cases that have already come to the attention of authorities. This is clearly an opportunity for improvement.

Taxa targeted for the illegal wildlife trade.

Reptiles were most targeted group of taxa in our study and were involved in 43% of prosecution cases from 1994 to 2007 (Figure 3.4), with 21 attempted exports, 22 attempted imports, and 12 cases where the species information was not available. Of the cases that involved reptiles, 24 were for illegal trade in snakes, 21 for lizards, 18 for turtles, and two cases where frogs and crocodile products were seized. Many seizures had a mixture of many different species (Australian Customs Service 2008c). Reptiles are favoured by illegal traders of wildlife because they can fetch large prices on the black market for pets, and are relatively easy to conceal and transport as live specimens. Reptiles have been found in a wide variety of places including packages in the mail (Australian Customs Service 2004d), concealed inside ornaments (Australian Customs Service 1999a), toys (Australian Customs Service 1999b), books (Australian Customs Service 2008b) and computer hardware (Australian Customs Service 2004a), wrapped in socks (Australian Customs Service 2002) and stockings, and stuffed in cigarette packets carried in a specially built vest (Australian Customs Service 2006b). The largest reported major seizure of reptiles in Australia occurred in 2003, in which a British national attempted to smuggle 219 reptiles and frogs out of Perth Airport, many of which were rare and endemic to the South-West of Western Australia (Australian Customs Service 2003a). In addition, and of considerable concern, several incidences involved the illegal importation of red eared slider turtles, *Trachemys scripta elegans* (Australian Customs Service 2006c). These turtles are a declared Class I pest under Queensland state wildlife legislation (*Land Protection (Pest and Stock Route Management) Act 2002*) because they can multiply rapidly and spread in waterways,

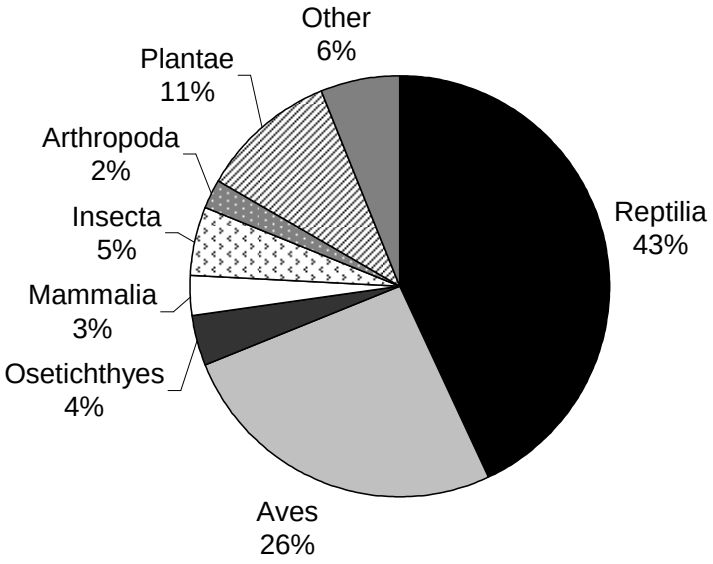


Figure 3.4 Types of wildlife involved in prosecutions from 1994 to 2007 including both illegal imports and exports from the Australian Customs Wildlife Prosecutions Database (Australian Customs Service 2008c).

probably compromising native turtles and other aquatic wildlife (Cadi, Delmas *et al.* 2004; Polo-Cavia, Lopez *et al.* 2008; Prevot-Julliard, Gousset *et al.* 2007).

Live birds and bird eggs are the second most common major seizure by the Australian Customs Service accounting for 26% of all case prosecutions (Figure 3.4). This conflicts with the report of the Senate Select Committee on the Commercial Utilization of Native Australian Wildlife (Anon 1998) which considered birds as the most common taxon involved in illegal wildlife trade, but we are unsure if this reflects a change in the composition of traded animals since 1998, or results from different methodologies. Certainly, live birds, rather than bird eggs, were more commonly seized prior to 2000 and this may have affected rates of detection. The high mortality rate of live birds during illegal international transport may have prompted a shift to smuggling of bird eggs because they have a lower mortality rate (although it is still considerable) and are easier to conceal under clothing vests specially made for this purpose. Australian parrots are highly sought after by overseas collectors with each parrot egg fetching up to $30,000. This demand is reflected by the high proportion of attempted exports of native birds and their eggs (62%), compared to only 24% for attempted imports (Australian Customs Service 2008c). For 15% of cases involving birds there was no information available as to whether it was an attempted export or import (these cases predate 1997). One of the largest seizures was an attempted export of 31 native bird eggs in 1995, estimated to have been worth more than $300,000 on the international market (Australian Customs Service 2008c).

The trade of wildlife products for sale as complementary medicines is prolific. In 2003, two shipping containers containing 160 kg of illegally imported wildlife products and body parts of endangered tiger, snakes, rhinoceros, Pangolin and an endangered plant (*Saussurea costus*) were found by Customs officers during a routine inspection (Australian Customs Service 2003b). It is impossible for Customs officers to inspect every shipping container that arrives in Australia and only a very small proportion of the shipments containing wildlife products are likely to be detected. In 2004, there was a crackdown on the sale of prohibited wildlife products with raids conducted by Customs, the Australian Federal Police, and DEWHA on five complementary medicine outlets in Sydney, Melbourne, and Brisbane. Large quantities of illegal imports containing products derived from endangered species were uncovered, including products labelled as bear bile, tiger bone and rhinoceros (Australian Customs Service 2004c).

There were also significant prosecutions for attempted illegal export of native flora, especially cuttings of native flowering plants, tree ferns, and orchids (Figure 3.4). Australia is a biodiversity hotspot for flowering plants, particularly in the South West of Western Australia. Many of these species are endemic, rare, and vulnerable to extinction from overexploitation (Hopper and Gioia 2004). Exotic fish are also occasionally smuggled into Australia and present a significant biosecurity threat to our aquaculture industries. Fish are often concealed in elaborate ways, such as in water-filled bags in padded luggage or, in one case, in water-filled plastic bags placed in pockets sewn into a specially-built unit worn underneath a skirt (Australian Customs Service 2007a). Other fauna that are frequently smuggled are insects, beetles, scorpions and spiders. These are usually smuggled through the mail system where the majority die during transit. Corals, ivory, hides, teeth, furs, and skins are also routinely confiscated by Customs officers (Australian Customs Service 2008c).

Impacts of illegal trade on Australian flora and fauna

The philosophy of the black market for wildlife where rare and endangered species are valued more than common species promotes the overexploitation in these rare species. Demand is driven by rarity, such that when a species becomes scarce the market value escalates making them even more attractive to collectors despite the greater effort required to collect specimens. This feedback process can rapidly drive species to extinction (Courchamp, Angulo *et al.* 2006). Listing of species for CITES and classification of species according to their level of vulnerability to extinction (*i.e.* vulnerable, endangered, or critically endangered) has been criticised by some experts because it may promote, as opposed to curb, the illegal trade in species by inadvertently advertising their rarity.

It is difficult to measure the direct impacts of illegal wildlife collections on wild populations of fauna and flora in Australia because it is probable that the majority of illegal harvests remain undetected. A nine year study of the broad headed snake, *Hoplocephalus bugaroides*, in Morton National Park, New South Wales, demonstrated that illegal collectors have seriously endangered the resident population. Rapid decline of the Morton National Park population in 1997 was most likely caused by increased illegal collection stimulated by an amnesty that allowed permits to be obtained for illegally collected broad headed snakes (Webb, Brook *et al.* 2002). Many more such studies are required to assess

the direct impacts of illegal harvests on rare and endangered fauna and flora of Australia. In addition, native flora and fauna can also be indirectly impacted by illegal trade resulting in the potential introduction of exotic pests and diseases (Normile 2004; Smith, Sax *et al.* 2006).

Penalties for illegal international trade of wildlife in Australia.

Fines were the most common penalty for wildlife case prosecutions between 1994 and 2007 (Figure 3.5). Fines are usually much less than the value of the wildlife goods on the international black market. The largest fine to date was $30,000 for the attempted exportation of 19 parrot eggs in 1998, only half of their estimated black market value of $60,000 (Australian Customs Service 2008c). In another case, in August 2005 a Japanese national was charged and fined $24,600 for an attempted smuggling of 24 long necked turtles (*Chelodina oblonga*), and a shingleback lizard (*Tiliqua rugosa*) via mail to Japan. Of the 24 turtles, 13 died during the attempt (Australian Customs Service 2005). Despite the hefty fine, it was considerably less than the estimated market value of the fauna. The turtle would typically sell for $1,400 and a shingleback for $4,000 in the Japanese black market, making the total seizure worth $37,600. Fines provide little deterrent to criminals, especially when they are less than the market value of the smuggled wildlife.

Less than one quarter (22%) of case prosecutions between 1994 and 2007 resulted in a prison sentence (Figure 3.5). The maximum sentence was three years and six months imprisonment for the importation into Australia of 20 exotic reptiles including six CITES II listed species (Australian Customs Service 2008c). The number of prosecutions which have received a prison sentence has not changed significantly since 1994 (data not shown), but the severity of the sentences has increased. There was an average of 10 months imprisonment for convictions between 1994 and 2003, compared to 28 months for convictions between 2004 and 2007. Good behaviour bonds have been issued in 13% of all convictions. In these cases, the defendant is released under strict conditions and non-compliance will result in imprisonment or a hefty fine.

Compared to the UK and US, Australia has tougher penalties for breaches against our wildlife legislation but the penalties that are actually issued for cases of illegal wildlife trade tend to be less severe. In the UK, the maximum sentence to date for wildlife trafficking is six years and six months imprisonment which was given for a case invloving

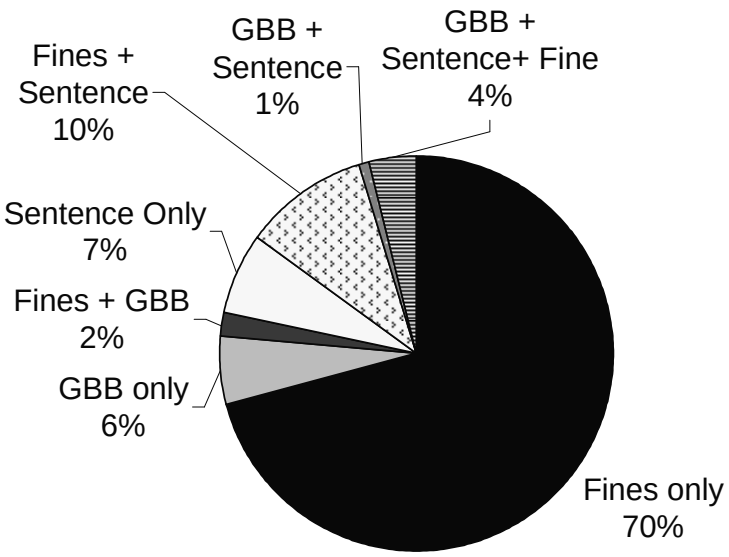


Figure 3.5 Types of penalties for wildlife case prosecutions from 1994 to 2007 reported by the Australian Customs Service Wildlife Prosecutions Database (Australian Customs Service 2008c). GBB refers to a penalty of a Good Behaviour Bond where the defendant is released under strict conditions and non-compliance will result in imprisonment or a hefty fine

22 counts of illegal trafficking of endangered species. The maximum fine issued by the UK was for £125,331 for 3 counts involving 126 rare and endangered orchids (PAW 2008). In the US perpetrators have been fined up $60, 000 for illegal trafficking of wildlife with 71 months imprisonment (Anon 2001). Although all cases differ and hence are not directly comparable the overall trends suggest that Australia's penalties for illegal wildlife trafficking have been less severe compared to the US and UK. Australia needs to adopt a tougher stance on the enforcement of its wildlife legislation by increasing the severity of fines and prison sentences to deter criminals from engaging in wildlife trafficking.

Tools to detect, and provide evidence for illegal wildlife trade cases.

Accurate identification of the specimen is critical for the investigation and prosecution of illegal wildlife trade cases, firstly to ascertain whether the seizure was native or exotic, and then to identify whether the specimen is CITES listed. Penalties may be more severe for CITES listed species, in accordance with the EPBC Act (1999), because these species are the most vulnerable to extinction from overexploitation for trade. Morphological examination by taxonomists or experts is usually sufficient for species identification of a specimen, but this can be impossible when specimens are highly processed (such as products commonly found in conventional medicines) or when distinguishing features are lacking (such as for bird eggs). Birds eggs can be incubated and hatched for species identification purposes but this is time consuming. Often the eggs are no longer viable because they have been crushed by the perpetrator or mishandled, and if the eggs are of an exotic species they can be a biosecurity risk. Stray feathers attached to the eggs can in some cases be used for species identification (Dove 1997), but these techniques have not been developed for the identification of Australian birds. Mammalian hairs also have characteristic microscopic characteristics that can be used for species discrimination (Cheng, Kang *et al.* 2007; Gonzalez-Esteban, Villate *et al.* 2006) and can be used to identify most Australian mammals and marsupials (*pers. comm. Silvana Tridico*). When feathers or hairs are not suitable for species identification or the products are finely processed such as in complementary medicines, DNA methods are ideal for species identification. DNA techniques were successfully used to provide evidence for a case in January 2007, in which 23 bird eggs illegally imported from Thailand into Sydney were identified to be two CITES II listed species; the African grey parrot (*Psittacus erithacus*) and the Electus parrot (*Eclectus roratus*), and one rare CITES Appendix I listed species, the Moluccan cockatoo (*Cacatua moluccensis).* These birds were valued at $250,000 on

the black market, and the defendant was subsequently convicted based on the DNA evidence, and sentenced to 2 years imprisonment and a $10,000 fine (Australian Customs Service 2007c).

In addition to identifying the species, DNA approaches can also be used to identify the geographic origins of a seizure. Identifying the geographic origins of a seizure can be used to distinguish between commercial trade and poaching (Wasser, Shedlock *et al.* 2004), identify areas where taxa are most vulnerable to illegal collection (Wasser, Mailand *et al.* 2007), and to repatriate seized animals and plants to their place of origin (Velo-Anton, Godinho *et al.* 2007). Unique DNA profiles can also be generated for individuals. These DNA profiles can be used to determine sex and verify the source of animals held by licensed breeders to ensure that the breeding stock is not being replenished or supplemented with illegal collections from wild populations. DNA profiles that characterise individuals have also been used to estimate the numbers of individuals traded in markets (Baker, Cooke *et al.* 2007). This technique may also be effective to estimate the numbers of animals that are used in various types of complementary medicines.

Future directions for wildlife forensics in Australia.

Wildlife crime in Australia is a low priority, and as a consequence much trade goes undetected. It is not commonly associated with organised crime, seen rather as the domain of individual transgressions, despite strong indications to the contrary. Severe restrictions on commercial trade reduce pressure from that quarter for effective policing, and a blanket ban on live wildlife exports, whether they be rare or common, engenders a public perception that concerns are largely to do with animal welfare. These are primary drivers likely to change over time and increase attention to wildlife crime in Australia, and the development of new more effective tools for regulation of legal trade and policing of illegal trade.

In the case of commercial activity, wild flowers and bush tucker are traded in a growing international market with public acceptance. Kangaroo leather and meat, emu meat, oil and crocodile products are also exported. Export of live native animals, however, is tightly restricted and commercial trade prohibited even for the ubiquitous budgerigar and cockatiel. One can anticipate increasing pressure for the commercial use of wildlife in Australia both under captive breeding programs and in the context of harvesting in the

wild. The Northern Territory Government has already moved in this direction, allowing harvest under licence of a wide range of native wildlife species for sale domestically, and a number of reports have evaluated the commercial and potential conservation benefits of trade in native wildlife and wildlife produces (Boulton 1997; McInnes 1998).

In 1998, the Senate Select Committee on the Commercial Utilization of Native Australian Wildlife (Anon, 1998) raised concerns that protectionist conservation practices are not working well and are expensive while covering only a small proportion of land. The Committee made a number of recommendations to explore a wider range of options for commercial use of native wildlife to achieve more satisfactory conservation outcomes off reserves.

This would include relaxing the tight restrictions against the export of live native species for species that are neither rare nor threatened. We can anticipate an increase in commercial trade in non-CITES wildlife as part of more general agreements on global free trade with attendant greater attention to the issues of regulation of that trade to protect wild populations, and increased pressure for more effective policing both domestically and at our borders. Legitimate commercial interests will demand greater policing of illicit trade where this undermines their profitability.

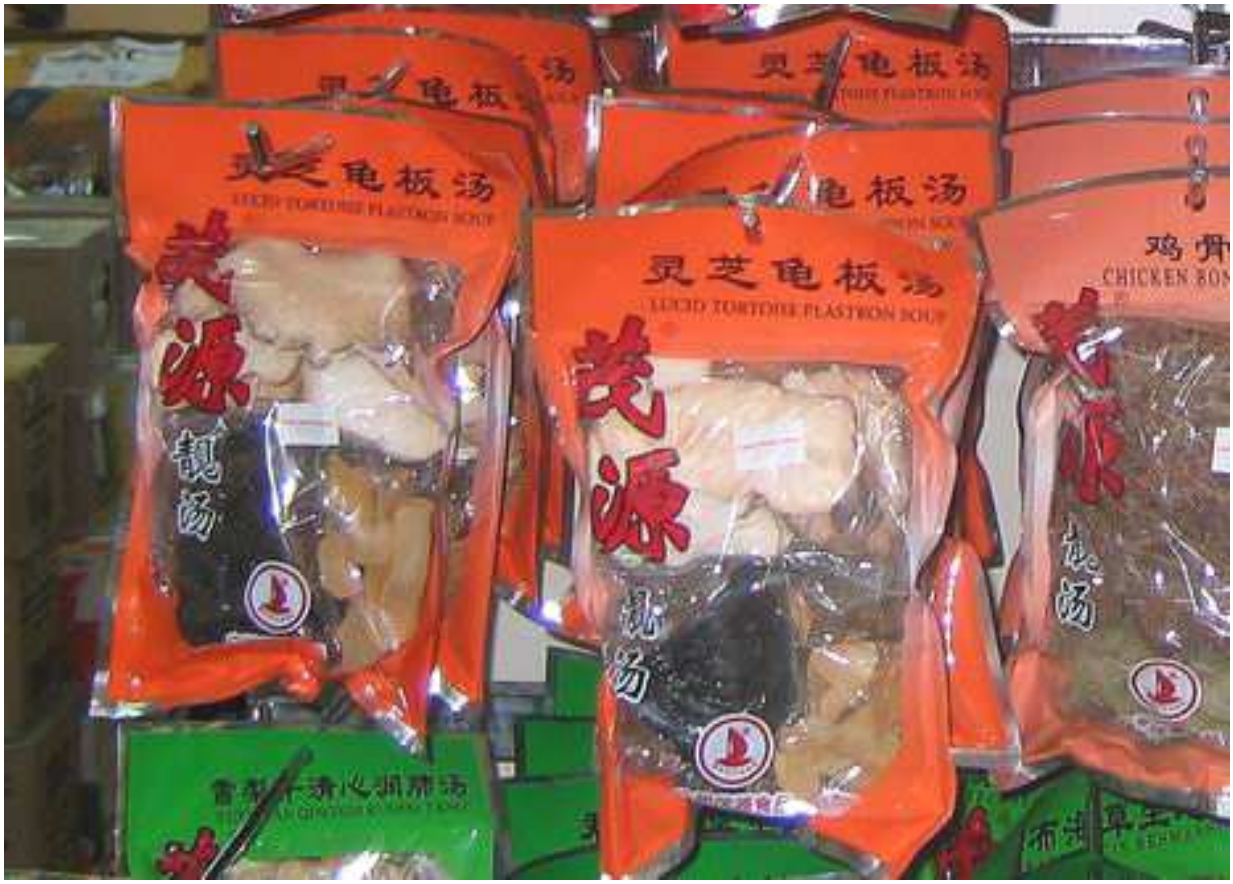
We can also anticipate improvements in DNA technologies with application to wildlife forensics, driven by the revolution in genomic knowledge and rapid screening techniques, are likely to see greater effectiveness of enforcement in the interests of both conservation of wild populations and commercial viability of legitimate enterprises. Over the past five years, the Australian Federal Police has funded several research programmes for the development of DNA technologies to provide evidence for crimes against Australian wildlife. These recent developments herald an exciting new era for wildlife forensics in Australia and if these efforts continue Australia may become one of the leading countries in the international effort to curb illegal trade of wildlife. The unique nature of Australian fauna and flora, and our geographic position, are such that our enhanced capability in this area is a critical and present need.

The degree to which DNA technologies can improve wildlife regulation and enforcement will depend on the structures put in place to pursue crime. The U.S. Fish and Wildlife

Service Forensics Laboratory located in Ashland, Oregon, U.S.A is the only laboratory in the world that is dedicated to crimes against wildlife. The laboratory is organized into seven operational units; administration, chemistry, criminalistics, genetics, morphology, pathology, and digital evidence. The administration unit is responsible for the processing of the evidence, maintaining chain of custody, and quality assurance. Crime scene investigation, fingerprint collection, fibre, bullet and glass comparisons are some of the diverse tasks carried out by the criminalistics units. Other units are involved in the provision of evidence including species identification of the seizure (chemistry, genetics, and morphology units), identification of gender and age (genetics and morphology units), analysing the composition of complementary medicines (chemistry), determining the cause of death of a seizure (pathology), and analysis of digital evidence (digital evidence unit). This team of specialists support the U.S. federal law enforcement of over 200 special agents and wildlife officers, as well as the 50 State Fish and Games Commissions, and all signatories of the CITES Treaty.

As a CITES member, Australia can receive support from the U.S. Fish and Wildlife Service but the expertise for Australian fauna and flora resides largely within the Australian scientific community. It is unlikely that the volume of wildlife related crimes would be sufficient to justify a wildlife forensic laboratory in Australia. Alternative solutions need to be explored. Some of these were canvassed at a workshop was held in Melbourne in October, 2007, with representatives and experts from the National Institute of Forensic Sciences (NIFS), Department of Environment, Water, Heritage and the Arts (DEWHA), Australia Customs Service, Australian Federal Police (AFP) Museums, and Universities to discuss the future of services for wildlife crime in Australia. A facility dedicated to wildlife-related crime was identified as a priority by all representatives. An Australian Wildlife Forensic Network (AWFN) was established to support, educate, and provide evidence for wildlife related crime in Australia. We argue that this initiative needs to be carried one step further, with Australia establishing a national clearing house for wildlife crime to deal with important issues to do with chain of custody, storage of forensic samples, and the coordination of a national network of experts to present the evidence and testimony.

Chapter 4 – A review of molecular approaches to wildlife forensics.



Picture: Traditional medicines made from turtle plastron sold openly in a shop in Phnom Penh, Cambodia. Photo by Jenny Daltry.

Chapter 4 – A review of molecular approaches to wildlife forensics.

Abstract

Illegal trade of wildlife is growing internationally and is worth more than USD$20 billion per year. Impacts of this trade on global biodiversity can be devastating and overexploitation for illegal trade is a major driver of species extinctions. The greatest challenges for enforcement agencies are to detect illegal dealings of wildlife and to obtain concrete evidence for wildlife convictions. DNA technologies are well suited to detect and provide evidence for cases of illicit wildlife trade and a suite of methods have been used for this purpose. Many of these methods have not been verified for forensic applications and the diverse range of methods employed can be confusing for forensic practitioners. In this review, we describe the various genetic techniques used to provide evidence for wildlife cases and thereby exhibit the diversity of forensic questions that can be addressed using currently available genetic technologies. We emphasise that the genetic technologies to provide evidence for wildlife cases are already available, but that the research underpinning their use in forensics is lacking. Finally we advocate and encourage greater collaboration of forensic scientists with conservation geneticists to develop research programs for phylogenetic, phylogeography and population genetics studies to jointly benefit conservation and management of traded species and to provide a scientific basis for the development of forensic methods for the regulation and policing of wildlife trade.

International wildlife trade and forensic genetics

According to Interpol (International Policing Organisation), the illegal trade of plants, animals and their by-products is a growing global black market commerce estimated to be worth more than USD $20 billion per year (Interpol, 2007). Organised international criminal networks have been linked to the trafficking of wildlife using their established drug smuggling routes to illegally transport wildlife across international borders (Cook, Roberts *et al.* 2002; Warchol 2004). In Brazil, recent estimates suggest that at least 40% of all illegal drugs shipments are combined with wildlife (Faiola 2002). Similarly, one third of all cocaine seized in 1993 was reported by the United States Fisheries and Wildlife Service (USFWS) to be associated with wildlife imports. The illicit wildlife trade is attractive to criminals because weight-for-weight wildlife is equally or more profitable than drugs or arms and with less associated risk. The rate of detection is lower and the penalties, if offenders are caught and convicted, are typically far more lenient for wildlife

crimes than for drugs or arms trafficking. Gaol sentences for wildlife smuggling are often minimal and fines disproportionately less than the commodity values of the goods on the black market (Alacs, Georges, 2008; Claridge *et al.*, 2005; Leader-Williams, Milner-Gulland, 1993; Li *et al.*, 2000). With little disincentive for criminal activity, the black market in wildlife continues to flourish, and places ever-increasing pressures on endangered species.

The illegal wildlife trade pose serious threats, both direct and indirect, to global biodiversity. Species sought for trade are directly impacted by over-exploitation. Over-exploitation is fuelled by the black market placing exaggerated values on rarer species. As a species becomes rarer from exploitation, its value on the black market escalates making it even more desirable despite the greater effort required to collect individuals from declining populations (Courchamp, Angulo *et al.* 2006). Over-exploitation of wild populations can rapidly cause local extinctions and, if harvesting is extensive across the range of the species, can cause global extinction. Widespread extinctions have occurred in taxonomic groups that are particularly vulnerable to the effects of overexploitation because of their life history characteristics such as longevity, high natural juvenile mortality, and low reproductive outputs. For example, turtles worldwide are in peril with 3% extinct or extinct in the wild, 9% critically endangered, 18% endangered, and 21% vulnerable (Turtle Conservation Fund, 2002). In Asia, the situation is even more dire with 1% extinct or extinct in the wild, 20% critically endangered, 31% endangered, and 25 % vulnerable (Turtle Conservation Fund, 2002). Over-exploitation of wild populations for meat, pets, and the use of the shells in traditional medicines are the major cause of declines in turtles worldwide, especially in Asia (Van Dijk, Stuart *et al.* 2000). Turtles are just one of many examples of taxa that are threatened globally from over-exploitation for trade. The list of species directly threatened by wildlife trade is extensive, encompassing all major taxonomic groups across all biomes. It includes many ‘keystone’ species (e.g. African horn bills, sea otters, grizzly bears, sea stars, elephants, orangutans, beavers, truffles and oysters), so named because they are ‘key’ to the functioning of the ecosystem and their loss causes widespread declines in many other species (Davic, 2003; Kotliar, 2000; Mills *et al.*, 1993). Direct exploitation for hunting, trade, and collection has been identified by the World Conservation Union (IUCN) as the second greatest driver (surpassed only by habitat destruction) of declines in endangered animals impacting 33%, 30% and 6% of threatened mammals, birds and amphibians, respectively (IUCN 2004). Wildlife trade also provides

avenues for the introduction of exotics with the potential to spread disease to native species (Lips *et al.*, 2006; Pedersen *et al.*, 2007; Skerratt *et al.*, 2007; Smith *et al.*, 2006; Spinks, Shaffer, 2007) or to become invasive (Keller, Lodge, 2007; Normile, 2004; Reed, 2005; Weigle *et al.*, 2005).

Monitoring trade in wildlife requires firstly the identification of the species traded, then assessment of whether they are derived from legal or illegal trade. Diagnostic morphological traits have traditionally been used as markers, but they are not suitable when traded products are degraded or highly processed as the morphological traits may not remain discernable. Molecular markers are ideal for species identification because unlike morphological markers they do not require intact specimens. DNA can be readily extracted from highly processed and degraded products commonly encountered in wildlife trade markets such as cooked and dried meats (Martinez, Danielsdottir, 2000; Wong *et al.*, 2004), claws left on tanned hides (Hedmark and Ellegren 2005), dried shark fins (Chapman, Abercrombie *et al.* 2003), egg shells (Moore, Bemiss *et al.* 2003), animal hairs (Branicki, Kupiec *et al.* 2003; Prado, Franco *et al.* 2002), bone (Prado *et al.*, 2002; Prado *et al.*, 1997), ivory (Wasser *et al.*, 2007; Wasser *et al.*, 2004), rhinoceros horns (Hsieh, Huang *et al.* 2003) and turtle shell (Lo, Lin *et al.* 2006).

Molecular technologies have great utility for wildlife forensics. Assigning geographic origins of trade products can also be achieved using molecular methods, a task that is often impossible using morphological traits alone. Knowledge of geographic origin can be used to distinguish between legal and illegal products, to assist in the repatriation of seized animals back to their source population, and to identify which populations are most intensively harvested for trade. At a finer resolution, individuals themselves can be marked and tracked using unique DNA profiles to characterise them. Additional information such as sex and parentage can also be ascertained which is especially useful for monitoring the compliance of registered breeders to wildlife regulations, such as to detect whether breeding stock has been supplemented or restocked with illegally caught wild stock.

In this review we detail the various contributions of genetics to wildlife forensics. The techniques employed for species identification, determination of geographic origin, individual identification and sexing will be briefly explained. Considerations for the application of these techniques to wildlife forensics will be discussed and illustrated with

published case studies. To conclude, we will describe new technologies on the horizon for wildlife forensics and the future role of genetics to combat the growing global black market dealing in wildlife.

Species identification methods

Several approaches have been adopted for identification of wildlife species distinguished by the DNA target (mitochondrial or nuclear) and the technique applied to develop the genetic marker (Table 4.1). Some techniques, such as sequencing, can be applied to investigate both types of DNA, while other techniques are specific to nuclear DNA (nDNA).

Mitochondrial DNA (mtDNA) is often favoured as a genetic marker over nDNA for species identification of wildlife because mtDNA is easier to type from highly processed and degraded tissue. This is because mitochondria are present in multiple copies per cell compared to one copy of nDNA from each parent (Randi 2000). Development time is typically substantially less for mtDNA markers compared to nDNA markers because universal mtDNA primers are available, which are used to amplify an informative segment of mtDNA across a wide range of taxa (Kocher, Thomas *et al.* 1989). Amplification is done using the polymerase chain reaction (PCR; Mullis, Ferré *et al.* 1994). Universal mtDNA markers have been successfully applied in the identification of wildlife for forensic cases. The most commonly used universal markers for species identification are the mitochondrial cytochrome b (*Cyt b*) and the cytochrome oxidase 1 (*CO1*) genes. Discrimination of species using a fragment of the *Cyt b* or *CO1* genes can be based directly on DNA sequence differences between species (Alacs *et al.*, 2003; Hsieh *et al.*, 2001; Lo *et al.*, 2006; Verma, Singh, 2003; Wong *et al.*, 2004) or by DNA profiling (discussed later). The *Cyt b* gene is an informative marker used in the identification of many vertebrate species from trade products including sharks (Chapman, Abercrombie *et al.* 2003), snakes (Yau, Wong *et al.* 2002), marine turtles (Lo, Lin *et al.* 2006), seals (Malik, Wilson *et al.* 1997) and tigers (Branicki *et al.*, 2003; Verma *et al.*, 2003; Wan, Fang, 2003). Sequencing of a 600 base pair (bp) portion of the *CO1* gene has been proposed to be an efficient, fast, and inexpensive way to characterise species and an international effort is underway to use this gene to catalogue all vertebrate biodiversity on earth (www.barcodinglife.org). Pyrosequencing is an alternative method for direct sequencing of DNA templates that uses a series of enzymatic reactions to detect visible light emitted during the synthesis of DNA

and enables more rapid screening of samples compared to conventional sequencing methods (Ronaghi, Uhlen *et al.* 1998). Only short fragments of 10 to 500 bp of DNA can be sequenced with pyrosequencing methods, which can limit its application in forensics unless highly variable and informative regions are targeted (Ronaghi, Uhlen *et al.* 1998). Karlsson and Holmlund (2007) used pyrosequencing to develop a highly sensitive assay to identify 28 species of European mammals based on short fragments of the mitochondrial 12S rRNA and 16S rRNA regions (17-18 bases and 15-25 bases respectively).

While mtDNA can be effective for species identification, it does have limitations that need to be considered and overcome before it can be used for forensic application. The matrilineal mode of inheritance of mtDNA may not reflect the patterns of nuclear genetic relationships between species particularly if there is strong sex-biased dispersal (Ballard and Whitlock 2004; Durand, Collet *et al.* 2005). Inheritance of the mtDNA genome can be complicated when paternal leakage results in heteroplasmy, that is, the coexistence of two or more different mtDNA genomes in the organism (Breton *et al.*, 2007; Kvist *et al.*, 2003; Rokas *et al.*, 2003; Sherengul *et al.*, 2006; Ujvari *et al.*, 2007). Nuclear paralogs (also called nuclear pseudogenes) of mtDNA genes occur when segments or the entire mtDNA genome inserts into the nucleus. These inserts can be subsequently subject to duplications, rearrangements or recombination and then may experience a different rate of mutation from that of their mtDNA parent (Arctander, 1995; Parr *et al.*, 2006; Thalmann *et al.*, 2004). Nuclear pseudogenes can be amplified simultaneously or even preferentially with the mtDNA gene in the polymerase chain reaction and the resultant mixture of genes with different modes of inheritance and mutation rate obscures any inferences of ancestry and evolutionary relationships of species (Behura, 2007; Podnar *et al.*, 2007; Spinks, Shaffer, 2007; Thalmann *et al.*, 2004). However techniques such as sequencing of the whole mitochondrial genome can be used to test for the presence of psedogenes and once they are accounted for they may even be phylogenetically informative (Behura *et al*. 2007; Triant DA 2009; Xu *et al.* 2009). Owing to lack of recombination (exceptions do occur, see Rokas *et al.*, 2003; Saville *et al.*, 1998) the mtDNA genome represents a single gene history and the evolutionary history of what is effectively a single gene may not accurately reflect the species history. Multiple genes, preferably both mitochondrial and nuclear genes, are recommended for species delimitation (Brower *et al.*, 1996; Maddison, 1997; Page, 2000; Sites *et al.*, 1996). Currently, markers derived from nuclear genes are not available for the majority of wildlife and consequently mtDNA approaches dominate

systems for species identification. However, the advent of whole genome sequencing of non-model organisms is expected to increase the availability of nuclear genes for wildlife in the next few years. If species have been delimited then mitochondrial data can serve, and has been used, as a robust tool for species identitification to provide evidence for forensic cases (An *et al.* 2007; Branicki et al. 2003; Cassidy and Gonzales, 2005; Ebach and Holredge, 2005). Mitochondrial techniques for species identification have been verified for use in forensic application (Branicki *et al.* 2003; Dawnay *et al.* 2007).

DNA profiles can also be generated using the technique of PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) to target specific areas of genetic variation among samples. Initially, the DNA segment of interest is amplified using PCR to generate millions of copies of the gene, and then subjected to digestion by restriction enzymes. These enzymes recognise specific base pair sequence motifs (that are often mirror images such as ATTA, GATTAG, etc) and cut the amplified fragment at these sites. Species that differ in base composition at the restriction enzyme recognition sites will differ in whether or not the enzyme cuts the DNA. This generates DNA fragments of differing lengths (i.e. polymorphic fragments), in which the number and size of the fragments depends on the number of cutting sites in the DNA fragment of interest (Upholt 1977). Electrophoresis of samples through an agarose or polyacrylamide gel separates fragments based on size and the different taxa will have characteristic banding patterns. Selection of restriction enzymes for PCR-RFLP analysis must ensure that the variability between species is appropriately represented and consequently that all species tested can be accurately discriminated from each other by their unique banding pattern, termed a RFLP profile. This technique has been successfully applied to a case in Argentina of a theft of livestock (Bravi, Liron *et al.* 2004) and for species identification of marine turtles (Moore, Bemiss *et al.* 2003). RFLP approaches are cheaper than direct sequencing and are suitable for forensic applications but they do not provide the baseline information that is required for the interpretation of forensic data such as the delineation of species boundaries.

For both direct sequencing and profiling approaches, phylogenetic studies (preferably with support from morphological, behavioural or physiological-based taxonomy) are necessary to form the foundation for accurate molecular species identification of wildlife. Phylogenetic studies estimate the evolutionary relationships of genes by inferring their common history and representing these relationships in the form of a phylogenetic tree.

More closely related genes are in closer proximity to each other on the gene tree compared to more distant relatives, with rooting of the tree at their implied most recent common ancestor (Vandamme 2003). These gene trees are used to infer the phylogenetic species, although this does require careful consideration because genes can evolve in an independent manner to the evolution of the species (Maddison 1997). It is also important to choose a gene with the appropriate mutation rate for phylogenetic analyses of species. A highly conserved gene will not be informative at the species level but will be better suited to the resolution of deep taxonomic relationships. Conversely, a gene that is evolving at a high rate will be informative at the level of the population or individual, but become saturated at the species level owing to homoplasy (Rosenberg 2002). Homoplasy arises when certain nucleotide sites are subject to repeated mutation over time, and mutations back to the original state occur, or when the same mutation occurs independently in different lineages. As a result two individuals can share a derived diagnostic base pair by chance rather than by descent (Sanderson and Shaffer 2002). When the appropriate gene region is chosen based on its rate of mutation, the levels of genetic diversity within and among species need to be sufficiently characterised before the gene can be applied to species identification of wildlife for forensic applications. This is to ensure that cryptic species – species that are morphologically indistinguishable but reproductively isolated – are represented (Bickford, Lohman *et al.* 2007). In addition, the biological characteristics of the species also need to be considered such as their propensity to hybridize. Hybridization between species is common for many groups of taxa (Arnold 1992; Dowling and Secor 1997). Owing to the predominantly maternal mode of inheritance of mtDNA, these phenomena might not be reliably detected in forensic samples with mtDNA markers alone particularly if there are sex-biased hybrid compatibilities (Ballard, Whitlock, 2004; Crochet *et al.*, 2003; Wang, Zhao, 2008; Whitworth *et al.*, 2007). A combination of mtDNA and nuclear markers (with their bi-parental inheritance) are recommended when hybridisation between species is suspected (Crochet *et al.*, 2003; Saetre *et al.*, 2001; Tegelstrom, Gelter, 1990; Tosi *et al.*, 2003).

Arbitrary fragments of genomic DNA (both mitochondrial and nuclear) have been used for species identification of wildlife with the major advantage that no prior genetic knowledge of the organism is required. One technique is termed Amplified Fragment Length Polymorphism (AFLP) and it generates segments of DNA of varying lengths that differ among individuals and species and can be visualised on polyacrylamide gels as series of

bands. To use the AFLP technique, genomic DNA is cut at specific sites using two restriction enzymes that target common sites and produce hundreds of fragments. A subset of these is specifically selected for PCR amplification and tagged with a fluorescent dye (Vos, Hogers *et al.* 1995). This allows their detection by a laser by gel electrophoresis on a polyacrylamide gel. AFLP profiling has been used to discriminate between illicitly cultivated marijuana and hemp (Alghanim, Almirall, 2003; Coyle *et al.*, 2003; Datwyler, Weiblen, 2006; Hakki *et al.*, 2003), identification of illegal hallucinogenic fungi (Coyle *et al.*, 2001; Lee *et al.*, 2000; Linacre *et al.*, 2002), and for the identification of species of legally protected owls and their hybrids (Haig, Mullins *et al.* 2004).

Compared to mitochondrial DNA, AFLP is better suited to the detection of hybrid individuals because of its biparental mode of inheritance (Congiu *et al.*, 2001; Nijman *et al.*, 2003). However, AFLP is not well suited for trace samples or highly degraded samples that are commonly encountered in forensics because it requires at least 50 – 100 ng of high molecular weight DNA (Bensch and Akesson 2005). Main sources of genotyping error for AFLP are differences in the peak intensities of loci between individual runs but error rates can be minimized by genotyping replicates for 5 to 10% of the samples and normalizing the peak height for loci against their average intensities (Bonin, Bellemain *et al.* 2004; Hong and Chuah 2003). A format for databasing and comparing AFLP profiles has been developed by Hong and Chuah in a user friendly software package which minimizes sources of genotyping error and shows great promise for use in the validation of the AFLP technique for forensic applications (Hong and Chuah 2003).

Sequence information from either mtDNA or nDNA can be used to develop species-specific PCR primers based on nucleotide differences between the aligned homologous sequences of the target species with other closely related taxa. These primers amplify DNA regions of the target species exclusively or have a PCR product of a characteristic size, and have been used to identify taxa of commercial interest or those commonly encountered in markets. Development of species-specific primer techniques first requires sequence data from all species likely to be encountered, for the design of putative primers. It is imperative that mixed genomic DNA samples are included in PCR tests of the development phase, to ensure that the primers are specific for the target species. Once developed, specific specific primer tests are a very rapid, sensitive and cost effective screening method to detect the presence of the target species in market products or from a

mixture of genomic DNA. Species-specific primers that amplify the nuclear *ITS2* region, and the mitochondrial *Cyt b* have been used to develop assays for the identification of various shark species from dried fins or meat (Chapman *et al.*, 2003; Clarke *et al.*, 2006; Magnussen *et al.*, 2007; Pank *et al.*, 2001; Shivji *et al.*, 2005). Where there is no prior sequence information available for the target species, RAPD (Randomly Amplified Polymorphic DNA – where arbitary primers are used to amplify random segments of DNA) or AFLP profiling can be used to generate species-specific primers (Negi, Devic *et al.* 2000). Bands specific to the target species are identified from the DNA fingerprint, extracted and sequenced. The sequence is used to design primers that will specifically amplify the species-specific region, termed a sequence characterized amplified region (SCAR). The SCAR method has the advantage of being highly reproducible: a shortcoming of the RAPD technique that has caused its redundancy in population genetic and forensic applications (Perez, Albornoz *et al.* 1998). Genotyping individuals with species-specific primers developed using the SCAR technique is considerably cheaper than sequencing or AFLP approaches, and has been successfully used to identify deer (Wu, Liu *et al.* 2006), snake (Yau, Wong *et al.* 2002), fish (Zhang and Cai 2006) and fly species (He, Wang *et al.* 2007).

Regardless of how species-specific primers are developed, more robust results are achieved using multiplex PCR where several primers are added to a PCR to simultaneously amplify different DNA regions of the target species in a single PCR reaction. To reduce the incidence of false negatives, universal primers that amplify across all potential taxa are included in the multiplex assay. If the universal primer amplifies but the species-specific primers fails to amplify the absence of the target species in the sample is confirmed. If both the universal and species-specific regions fail to amplify, the PCR reaction is deemed not successful and the result is inconclusive. Multiplex PCR reactions may also be used to identify several different species in a single assay. For example a multiplex PCR with six different species-specific primers and two universal shark primers for positive controls has been used to identify six species of sharks commonly encountered in North Atlantic fisheries (Shivji, Clarke *et al.* 2002). Species-specific primers are ideal for forensic applications because they are cost effective and can be used for large scale screening of samples. However, a considerable amount of development time is required and once developed it can be difficult to incorporate additional species in the multiplex assays. Furthermore a comprehensive understanding of species boundaries is required prior to the

development of species-specific primers to ensure that all species likely to be encountered, including cryptic species, are included in the assay and can be reliably distinguished from each other.

Identification of a forensic specimen to its geographic origin

In many wildlife forensic cases, such as where commercial trade is established, the identification of the traded product or 'specimen' to the species level may not be sufficient and the geographic origin needs to be determined. Geographic origins of an individual can be identified if there is known genetic structure within the region of interest using phylogeography or population assignment methods. Phylogeographic studies assess the geographic distribution of genealogical lineages where specific mtDNA haplotypes are associated with broad geographic regions (Avise, Arnold *et al.* 1987). For example, phylogeographic data for four species of seahorse (*Hippocampus barbouri*, *H. spinosissimus*, *H. trimaculatus*, and *H. ingens*) were used successfully to determine the broad geographic origins of seahorses that were found for sale in traditional medicine and curio shops in California (Sanders, Cribbs *et al.* 2008).

More subtle genetic differences can often be detected using population assignment methods in comparison to phylogeographic analyses. Population assignment methods are based on allelic differences at hypervariable nDNA genetic markers between groups of individuals, also loosely called 'populations'. Assignment tests are used to estimate the probability of an individual belonging to each of these putative populations, and the forensic specimen is 'assigned' to its most probable population of origin. Conversely, exclusion tests can be used to reject the hypothesis that a specimen originated from a particular population (Cornuet *et al.*, 1999; DeYoung *et al.*, 2003; Gomez-Diaz, Gonzalez-Solis, 2007; Manel *et al.*, 2005). The hypervariable markers most often used for population assignment or exclusion are AFLPs, and microsatellites (also called simple tandem repeats or STRs). Microsatellites are short sequence motifs typically 1-6 nucleotides in length (e.g., ATATATAT) that have a high mutation rate predominantly due to slippage of the polymerase during DNA replication (although other mutation mechanisms have been proposed, see Ellegren 2004) resulting in lengthening or shortening of the number of repeat units. Microsatellites are codominant markers in which the gene variants (or alleles) inherited from both parents are amplified in a PCR reaction and visualised on a polyacrylamide gel (Figure 4.4.4). Homozygote individuals have the same sized STR

repeats (e.g., $[AT]_6$, $[AT]_6$) whereas heterozygote individuals have different sized repeats (e.g., $[AT]_3$, $[AT]_6$). In contrast AFLPs are dominant markers where an allele (or fingerprint band) is either present in the individual or absent. The heterozygosity of an individual cannot be determined directly from an AFLP band (or locus) and hence these dominant markers have far less resolving power per locus to determine the population origins of an individual in comparison to the codominant microsatellite markers (Bensch, Akesson, 2005; Campbell *et al.*, 2003). Typically, at least eight microsatellite loci or 50 AFLP loci are recommended for population assignment studies (Campbell, Duchesne *et al.* 2003). The AFLP technique requires high quality DNA and hence is less versatile for degraded or trace samples in comparison to microsatellites (Campbell, Duchesne *et al.* 2003). The genotyping errors associated with AFLPs have been found to be greater than for microsatellites mainly because of differences in peak height intensity (Bonin et al. 2004).

A suite of statistical analyses for assignment methods are currently used to identify the origins of individuals based on their AFLP or microsatellite profile, with the most suitable method depending on the scenario (Campbell *et al.*, 2003; Cornuet *et al.*, 1999; Duchesne, Bernatchez, 2002; Evanno *et al.*, 2005; Manel *et al.*, 2007; Manel *et al.*, 2005; Maudet *et al.*, 2002; Paetkau *et al.*, 2004; Piry *et al.*, 2004; Waples, Gaggiotti, 2006). Assignment tests are highly accurate when all potential source populations have been sampled, populations boundaries are well defined, sampling is random, and populations are in Hardy-Weinberg equilibrium (i.e. there is a balance between mutation and genetic drift, no inbreeding and random mating). However, these assumptions are not realistic for many populations such as when populations are small, population boundaries are not clear or the genetic divergences between populations are low (Manel, Berthier *et al.* 2002). For population with ill-defined boundaries, clustering methods perform well because they can determine the number of populations (i.e. clusters) present based on the multilocus genotypes of individuals rather than on pre-determined boundaries. They then assign individuals to these identified populations, including to populations that have not been sampled (Mank and Avise 2004). Programs such as Geneland (Guillot, Mortier *et al.* 2005) can map the probabilities of an individual belonging to a ‘cluster’ or ‘population’ onto the landscape in an easily interpretable visual format ideal for the presentation of evidence to a jury in a court of law. Other methods such as spatial smoothing are most effective when

the organism has a continuous distribution across the landscape and a spatial structure is not imposed (see Manel, Gaggiotti *et al.* 2005 for review of assignment methods).

For example, spatial smoothing assignment has been successfully used to monitor the African elephant ivory trade by characterisation of the allele frequencies of 16 STR loci across the entire African elephants' range. Geographic-specific alleles were shown to be effective in the inference of the geographic origin of individual DNA samples with 50% identified to within 500 km of their source, and 80% to within 932 km of their source (Wasser, Shedlock *et al.* 2004). This study was later applied to a forensic case involving the largest seizure of contraband ivory since the 1989 ban on the ivory trade. A total of 532 ivory tusks, and 42,210 "hankos" which are ivory cylinders cut from the solid portion of the tusk, were found in a container shipped via South Africa to Singapore in June 2002. Assignment tests using the 16 STR loci indicated that the ivory was entirely from savannah rather than forest elephants and most likely originated from a narrow strip of southern Africa that centred on Zambia (Wasser, Mailand *et al.* 2007). This information is invaluable for wildlife enforcement agencies to identify current poaching "hot spots" and to identify whether legally declared government stockpiles are being illegally traded and replenished (Wasser, Mailand *et al.* 2007).

Assignment tests have also been used to relocate seized animals of unknown origin back to their original population. European pond turtles (*Emys orbicularis*) are highly sought after for pets and hence are often subject to illegal collection. Specimens seized by wildlife authorities are sent to recovery centres or zoos where they rapidly accumulate in large numbers. When the turtles become too numerous to maintain in these facilities they are sacrificed or re-located to their supposed region of origin. Characterisation of three turtle populations at seven microsatellite loci assigned 22 of 36 turtles in recovery centres to their population of origin (Velo-Anton, Godinho *et al.* 2007). Releasing turtles that have been genotypically assigned to the population reduces the risk of outbreeding depression, which is a reduction in reproductive output and fitness that can result when two genetically distinct populations interbreed. Such targeted releases also minimize the possibility of corruption of the evolutionary processes leading to divergence among geographic isolates, an important precursor to speciation.

In a similar manner to population assignment tests, exclusion tests can be used to exclude individuals as belonging to a given population based on their allelic or genotype frequencies. An example of the use of exclusion tests to provide evidence for a wildlife related crime is the 2004 case of a suspected illegal translocation of four red deer (*Cervus elaphus*) into a hunting area in Luxembourg. Exclusion tests based on allelic frequencies for 13 microsatellite loci verified that the Luxembourg red deer were not founded from migrants from the adjacent populations of France, Belgium and Germany. Instead, they were most likely sourced from deer farms and had been illegally translocated into the area for recreational hunting (Frantz, Pourtois *et al.* 2006). Genotype exclusion tests, based on ten microsatellite markers, have also been used to successfully discriminate between hatchery-raised versus wild stocks of the commercially important marine fish red drum, *Sciaenops ocellatus,* of the south eastern United States (Renshaw, Saillant *et al.* 2006).

Individual identification, sexing, and parentage

Identification of an individual based on their unique genetic profile can be used to monitor the number of animals entering commercial markets, even if they are sold as meat or highly processed products. Baker *et al.* (2007) combined market surveys with DNA profiling to estimate the numbers of North Pacific minke whales (*Balaenoptera acutorostrata spp*.) sold in 12 markets in the Republic of (South) Korea from 1999 to 2003. A 464 bp fragment of the mtDNA control region and eight STRs were used to develop a 'DNA profile' for each market product. The DNA profiles were evaluated for matches with other profiles and the numbers of unique DNA profiles were assumed to be minimum number of individual whales sold on the market, with matching DNA profiles representing replicates from the same individual. The total number of individual whales sold over a five period was estimated to be 827, almost double the officially recorded by-catch of 458 whales during this period suggesting that illegal trade of North Pacific minke whales in South Korea is rampant (Baker, Cooke *et al.* 2007). Characterising individuals with unique DNA profiles is an accurate method of monitoring markets to determine what species are present, and the numbers of individuals of each species sold.

To distinguish between legally and illegally obtained specimens, a DNA register can be established where each legal specimen is DNA profiled in a certified laboratory and the profiles are lodged in a database. When there is a confiscated specimen, the DNA database can be interrogated to rapidly identify unregistered (and presumably illegally obtained)

specimens. In Norway, a DNA register for minke whale has been established containing 2676 individual genetic profiles. The genetic profiles are generated using information from the mitochondrial control region, two sex determination markers and 10 microsatellite loci. The Norwegian minke whale DNA register has proven to be effective in verifying legal specimens by consistently matching 20 specimens of minke whales obtained from Norwegian markets to reference samples in the register (Palsboll, Berube *et al.* 2006). An effective DNA register requires all legal specimens to be lodged and genotyped using highly sensitive hypervariable markers, such as microsatellites, that have the resolution to differentiate between individuals. Wildlife DNA registers can also be used to monitor the compliance of breeders to ensure that captive bred stock are not being replenished or supplemented from illegally wild caught stock. The offspring of captive breeding stock can also be verified by assessing the parentage using a suite of hypervariable microsatellite markers, similar to methods used for human paternity analyses (Cassidy and Gonzales 2005).

Determining the sex of an animal can be difficult for some taxa where differences between the sexes are not obvious or the illegally killed carcass is decomposed. Determining the gender of the Asian elephant is important because tusks are only present in males and drastic declines in the numbers of males from hunting for their ivory can result in unbalanced sex ratios in the population. It can be difficult or impossible to determine the sex of Asian elephants when the carcass is decomposed, but a simple and inexpensive test based on the SRY gene on the Y chromosome has been developed specifically for identifying male Asian elephants from poached carcasses (Gupta, Thangaraj *et al.* 2006).

In some countries, qualified hunters are restricted to hunting only one sex and monitoring the trade requires determination of the sex of the animals hunted. In Korea the hunting of female pheasant is illegal and sex-specific markers have been used to identify illegal hunting. In one case (February 2004), five pheasant carcasses were found in a suspect's refrigerator. Using two avian sex-specific markers, one marker on the Z chromosome and one on the W chromosome, gender could be determined because avian males are homozygotes (ZZ), whereas females are heterozygotes (ZW). Two of the five pheasant carcasses were female and the suspect was subsequently prosecuted for illegal hunting based on the DNA evidence (An, Lee *et al.* 2007).

Method Validation

Genetic techniques need to be validated for use in forensic applications. This is not a trivial matter either at a scientific level or at a legal level. The use of DNA markers for wildlife forensic application need to be tested against what has become the 'gold standard' for forensic science, the validation of human DNA. Butler (2005) defines validation as the process of demonstrating that a laboratory procedure is robust, reliable, and reproducible in the hands of the personnel performing the test. A robust method is one in which successful results are obtained in a high percentage of tests at first testing. A reliable method is one in which the results are accurate and correctly reflect the sample being tested and a reproducible method is one in which the same result is obtained each time a sample is tested. All three properties are important for techniques performed in forensic laboratories (Butler 2005). To meet these exacting standards, forensic scientists need to document (a) full details of the tests used to validate new techniques; (b) the technical procedures and policies to instil confidence in the laboratory processes and policies; and (c) the policies relating to the interpretation of data.

In the forensic world, most DNA analysis is conducted using commercially available technologies, reagents and 'kits'. The **development validation** of the latter will have been carried out by the commercial entity prior to product release. Hence, the forensic laboratory is required to carry out more limited **internal validation** aimed at showing the laboratory can meet accepted validation requirements (Butler 2005).

Development validation is an exacting process and several organisations at an international level have defined the standards for forensic application. The most commonly used standards are these developed by SWGDAM (Scientific Working group on DNA Analysis Methods). This group was first established in the late 1980's under FBI sponsorship to aid forensic scientists as DNA applications in forensics first emerged. A detailed discussion of these and similar guidelines is beyond the scope of this paper, but they include testing the technology for consistency and reproducibility against standard samples, samples in more complex matrices, mixed samples and samples exposed to a variety of environmental conditions. These criteria are aimed at ensuring the technologies are robust in producing reliable results with real life forensic samples. SWGDAM also makes recommendations

with regards to population studies and data interpretation (Scientific Working group on DNA Analysis Methods 2004).

It can readily be appreciated that forensic validation studies are onerous and time consuming. There have been few comprehensive attempts to validate non human DNA tests for forensic applications. One recent major study validated DNA markers for Cannabis plant samples (Howard, Gilmore *et al.* 2008) and this work gives a useful insight to the challenges which will face scientists seeking to cross the bridge between research and development to professional application in forensics.

The admissibility of evidence in the legal system is governed by different rules which also need to be met. These vary according to the legal frameworks and systems in different countries. In much of the United States, scientific evidence must meet the Daubert standards (Girard 2008), under which scientific techniques must have

- been tested before;
- been subject to peer review and publication;
- standards which can verify the reliability of the technique;
- known potential error notes; and
- gained widespread acceptance in the scientific community.

Scientists seeking to bring wildlife forensics into the court system need to be aware of the legal framework and rules, and the role and expectations for the expert witness. These are not trivial matters.

Future Directions of Genetic Markers in Wildlife Forensics

The advent of whole genome sequencing of non-model organisms will greatly increase the markers that are currently available for forensic genetics of wildlife. Universal nuclear primers that can amplify informative regions over a broad range of taxa will become more readily available, and will complement current initiatives such as the mtDNA barcoding of life project (www.barcodinglife.org). In addition, the availability of single nucleotide polymorphisms (SNPs) that are informative for species identification, population assignment, and individual identification of wildlife will increase considerably. SNP techniques target multiple regions of the genome where single base pair mutations have

occurred and they have the major advantage compared to other forensic genetic methods of being easily amplifiable from highly degraded material and are highly reproducible across different laboratories (Amorim, Pereira, 2005; Budowle, 2004; Chakraborty *et al.*, 1999; Sarkar, Kashyap, 2003). Furthermore, SNPs are amenable to multiplexing of up to 50 loci on a microarray platform enabling rapid and high throughput screening of forensic samples (Divne and Allen 2005). SNPs have already proven to be effective for forensic identification of the population of origin for Chinook salmon (Schwenke, Rhydderch *et al.* 2006) and show great promise as a genetic marker to contribute to existing forensic genetic technologies.

Whilst emerging technologies will add to the forensic genetic toolbox, current genetic technologies are capable of addressing most forensic questions as evidenced by the suite of methods discussed in this review which have been successfully applied to wildlife forensic cases. The choice of genetic marker will depend on the forensic question to be addressed and the ecology, biology and genetic knowledge of the species. Each genetic technique has it advantages and limitations for forensic applications and these must be carefully evaluated when choosing a marker (Table 4.1). To overcome the limitations of the genetic techniques the most powerful approach is to use a combination of complimentary markers with the appropriate resolution to address the forensic outcomes.

Ecological, biological, and genetic knowledge of wildlife has traditionally been covered in the disciplines of wildlife ecology, physiology and conservation genetics and it is this research that forms the foundation for the interpretation of genetic data for forensic applications. We argue that for forensic science to advance in the field of wildlife, cross-disciplinary collaborations with ecologists, biologists and conservation geneticists are essential.

Phylogenetic, phylogeographic and population genetic studies are required for species, population and individual identification of wildlife respectively. The objectives of conservation research are often complimentary with forensic outcomes. For example, phylogenetics can be used to delineate species boundaries and this is important for the enforcement of wildlife legislation which recognises and protects groups that are classed as ‘species’ or ‘subspecies’. In addition, the markers developed for phylogenetics can also be used, or modified for use, for forensic species identification (Table 4.1). Phylogeography

and population genetic studies not only provide baseline data that is required for assignment of individuals to their geographic source of origin, but are also used to identify populations that are most vulnerable to extinction from overexploitation. The markers used for these same studies can be applied to elucidate the source of traded specimens and thereby identify “hotspots” for illegal collection where enforcement efforts can be directed.

Forensic scientists can greatly benefit from liaison with conservation geneticists to collaboratively develop genetic technologies that will benefit the conservation and management of traded species and to extend these technologies for use in a forensic context to monitor trade activities and provide DNA evidence that can be presented in court for cases of illicit trade of wildlife. Cross-disciplinary collaboration in the initial planning phase of the research programmes will foster the development of new technologies that have greater versatility with applications for both conservation and forensics.

Table 4.1 Comparison of genetic markers used for forensic applications. √√ is highly informative, √ informative, × not informative.

	Species Id	Regional Id	Population Id	Individual Id	Parentage	Limitations for Forensics	Advantages for Forensics	Applications to generate baseline genetic data.
Mitochondrial gene (mtDNA) sequencing	√√	√√	√	×	√√ maternity × paternity	・Heteoplasmy ・Nuclear paralogs ・Maternal inheritance ・Single linked genome hence effectively is one single marker	・Suitable for trace and degraded DNA ・Universal primers available	・Phylogenetics ・Phylogeography ・Population genetics
Nuclear gene (nDNA) sequencing	√√	×	×	×	×	・Not suitable for trace or degraded DNA ・Universal primers not available for most species	・Recommended for use in combination with mtDNA for species ・identification ・Can detect hybrid individuals	・Phylogenetics

Table 4.1 continued Comparison of genetic markers used for forensic applications. √√ is highly informative, √ informative, × not informative.

	Species Id	Regional Id	Population Id	Individual Id	Parentage	Limitations for Forensics	Advantages for Forensics	Applications to generate baseline genetic data
Pyrosequencing	√√	√ not assessed	√√ for SNP genotyping	√√ for SNP genotyping	√√ for SNP genotyping	・ Only short fragments of 10 to 500bp can be sequenced.	・ Enables very rapid high throughput genotyping of short fragments or SNPs	・ Population genetics for SNP genotyping
Amplified Fragment Length Polymorphism (AFLP)	√	√	√	√	√	・ Dominant marker, therefore less informative for all applications. ・ Not suitable for trace or degraded DNA	・ No prior genetic knowledge of the organism required.	・ Phylogenetics, phylogeography population genetics. Limited use because of their dominance.
Species-specific Priming	√√	×	×	×	×	・ Knowledge of species boundaries required	・ Rapid screening once developed. ・ Cost effective.	・ None

Table 4.1 continued Comparison of genetic markers used for forensic applications. √√ is highly informative, √ informative, × not informative.

	Species Id	Regional Id	Population Id	Individual Id	Parentage	Limitations for Forensics	Advantages for Forensics	Applications to generate baseline genetic data.
Short Tandem Repeat (STR). Also called Simple Tandem Repeat (SSR) or microsatellite.	×	√√	√√	√√	√√	・Allelic dropout can occur when trace or degraded DNA is used. ・Development time is substantial.	・Highly informative marker for many applications. ・Techniques have already been validated for human forensics.	・Most commonly used marker for population genetics because of its high information content.
Single Nucleotide Polymorphism	√√	√√	√	√	√	・Approx five times more loci required compared to STRs. ・Currently not available for many species.	・Highly reproducible. ・Rapid screening of samples	・Use of this marker for phylogenetics, phylogeography and population genetics is still in its infancy.

Chapter 5 - Development of microsatellite markers in the Australasian snake-necked turtle *Chelodina rugosa*, and cross-species amplification.

Picture: *Chelodina canni*: one of the eight species that amplification of the microsatellite markers was tested in. Photo by Erika Alacs.

Chapter 5 - Development of microsatellite markers in the Australasian snake-necked turtle *Chelodina rugosa,* and cross-species amplification.

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Abstract

Seventeen microsatellite loci were developed for the snake-necked turtle, *Chelodina rugosa* (Ogilby, 1890). Sixteen of the loci were polymorphic but three of these loci had null alleles. One locus displayed linkage disequilibrium. These 17 markers were tested for amplification in eight congeneric species with varying success; 98% amplification in *Chelodina burrungandjii*, 72% in *C. canni*, 38% in *C. expansa*, 58% in *C. longicollis*, 67% in *C. mccordi*, 73% in *C. oblonga*, 81% in *C. parkeri*, and 68% in *C. pritchardi*. These microsatellite markers will be useful for population assignment, gene flow, mating systems and hybridization studies in the genus *Chelodina.*

Main Document

The genus *Chelodina* (Testudines: Chelidae) is an Australasian genus of snake-necked turtles comprising thirteen species from Australia, Papua New Guinea, East Timor, Indonesian West Papua and Roti (Georges, Thomson 2006). *Chelodina rugosa* is found in parts of northern Australia and southern Papua New Guinea and is subject to legal harvest for the pet trade by the indigenous community of Maningrida in Arnhem Land, Northern Territory. We developed 17 microsatellite loci to test whether we could distinguish between legal collections of *C. rugosa* in Arnhem Land and illegal poaching activities. These loci were characterised for 76 individuals from two populations of *Chelodina rugosa* from the Northern Territory that are 1.2 km apart (sample sizes of 41 and 35 respectively). We also tested the primers on eight other species: *Chelodina burrungandjii, C. canni, C. expansa, C. longicollis, C. mccordi, C. oblonga, C. parkeri* and *C. pritchardi* to better understand the complex patterns of hybridisation that occur in this genus (Georges *et al.* 2002).

Total genomic DNA was extracted from skin tissue samples (taken from vestigial hind toe webbing) using standard salting-out protocol (Dethmers *et al.* 2006). A genomic library enriched for di- and trinucleotide repeats was constructed based on the FIASCO method (Zane *et al*., 2002). Modifications on the prescribed method are described below. DNA from a composite sample of four individuals (approximately 100ng) was simultaneously digested with *Mse*I and ligated to *Mse*I AFLP adaptor (5´- TACTCAGGACTCAT-3´ / 5´- GACGATGAGTCCTGAG-3´). The subsequent digestion-ligation mixture was amplified using Polymerase Chain Reaction (PCR) under standard cycling conditions with the primer *Mse*I-N (5´- GATGAGTCCTGAGTAAN-3´). Amplified DNA was hybridised with a 'pool' of biotinylated probes ($(AAC)_8$, $(ACC)_8$, $(AGC)_8$, & $(ACG)_8$) by mixing preheated hybridisation buffer (181 µl 6 × SSC, 3 µl 10% SDS, 6 µl 50 × Denhards) with a denatured solution containing 100 µL of amplified DNA and 10 µL of the probe pool. The total solution was incubated at 62 ^{o}C for 30 mins. Hybridized DNA molecules were selectively captured using Streptavidin MagneSphere Paramagnet Particles (S-PMP) (PROMEGA). 200µL 6× SSC, 4µL 50× Denhards and 2µL 10% SDS, were added to the S-PMP, followed by the prepared DNA-probe hybridization and rotated for 20 mins. The resultant S-PMP-probe-DNA conglomerate was then isolated using magnetic field separation. Removal of non-specific DNA occurred through a sequence of two non-stringency washes followed by four stringency washes. Non-stringency washes were performed with gentle mixing for 30 secs using 6 × SSC, and 2 × SSC, respectively. Stringency washes were performed using 0.99 × SSC, 0.1% SDS; the third and fourth washes were rotated at 55 ^{o}C for 5 minutes and 30 mins, respectively. Between each wash, DNA was recovered by magnetic field separation for 3 mins. The enriched DNA was resuspended in 40 µL ddH_2O. One microliter of enriched DNA was amplified using the same conditions as for the pre-hybridisation PCR. Fragments of 200-1000 bp were excised from a 1.2% agarose gel, purified and ligated into PGEM-T easy vector, using T4 DNA Ligase (Promega). Ligations were used to transform competent *E. coli* cells (strain JM109, Promega) through electroporation. Following electroporation, the methods for cloning, identifying and subsequently extracting DNA from positive clone colonies followed those described in Hillyer *et al.* (2006). Sequencing of 90 clone colonies used the M13 F primer (5´- GTAAAACGACGGCCAGT -3´) (Amersham Pharmacia Biotech) and Big Dye Terminators (Perkin Elmer). Sequences were determined on an ABI 377 automated sequencer and edited by eye in BioEdit (Version 5.0.9: Hall, 1999). Of the sequenced

clones, 55 contained microsatellite arrays, 31 of which had sufficient flanking regions allowing for primer design.

Primer pairs for up to seventeen loci were first tested on five individuals of *Chelodina rugosa* to select those that successfully amplified and were polymorphic. The remaining 14 loci were not tested for polymorphism in *C. rugosa*. PCR primers were designed with 5' fluorescent modifications (WellRed dyes) to allow PCR multiplexing for up to six loci (Table 5.1). Each PCR amplification was performed in a 20 µl reaction containing 50–100 ng template, 2 × PCR buffer, 2–2.5 mM $MgCl_2$ (Table 5.1), 0.2 mM each dNTP (Bioline), 0.10–0.75 μM of each primer (Table 5.1) and 1 U of *Taq* DNA polymerase (Bioline RedTaq). All reactions had an initial five minute denaturation at 94 ^{o}C, followed by 30 cycles of 94 ^{o}C for 1 minute, annealing temperature (T_a) for 30 seconds (Table 5.1), and 72 ^{o}C for 1 minute, with a final extension of 4 minutes at 72 ^{o}C. DNA fragments were separated on a Beckman Coulter CEQ 8000 Genetic Analysis System and sized with Beckman Coulter version 9.0 CEQ software using 400 DNA ladder as an internal size standard.

Characteristics of the 17 loci are summarized in Table 5.1. The locus T27 was monomorphic for *Chelodina rugosa* but was polymorphic in other species (Table 5.2). Expected and observed heterozygosity, and the number of alleles per locus were generated using POPGENE 1.31 (Yeh *et al.* 1999). Each locus was tested for deviations from Hardy-Weinberg equilibrium and linkage disequilibrium using GENEPOP 3.4 for each population separately and all samples combined (Raymond, Rousset 1995). Micro-Checker 2.2.3 (Van Ooserhout *et al.* 2004) was used to detect null alleles in each population and the program FreeNA (Chapius, Estoup, 2007) was used to estimate the frequency of the null alleles. Linkage disequilibrium was found between loci T26 and T41, and between T26 and T17 for both populations. Significant deviations from Hardy-Weinberg equilibrium were found for loci T15, T39, and T67 ($p < 0.05$), in which observed heterozygosity was lower than expected heterozygosity. Micro-Checker analyses detected possible evidence for null alleles, and null allele frequencies of 11.8% 12.9% and 9.8%, respectively, suggesting there are null alleles at these loci (Table 5.1). Dinucleotide microsatellite loci displayed greater allelic richness (mean $N_A = 13.8$) compared to trinucleotide loci (mean $N_A = 4.4$) but the levels of expected heterozygosity were both high (mean $H_e = 0.69$ and 0.43 for

Table 5.1 Characterisation of 17 microsatellite loci for the Australasian turtle,*Chelodina rugosa* for 76 individuals.

Locus	Primer sequences (5' → 3')	Repeat Motif	PCR primer (μM)	T_a (°C)	$MgCl_2$ (mM)	Allele size range (bp)	N_A	N_E	H_o	H_E	Null Freq.	GenBank Accession no.
T-11[c]	F[3]: CAG CCA AAA AAA TGT AGG TCC R: TGT GAC CAC CTG ATA ACA GGC	$(CA)_{24}$	0.25	57	2	143-209	11	5.1	0.800	0.817	0.016	EU522102
T-12[a]	F[2]: GGG ATC ACT CGG CCA CTC TGG R: ACC CAA GAA TAC CCG TCA CCG	$(CAG)_9GAG(CAG)_3$	0.40	57	2	157-163	3	2.4	0.514	0.591	0.011	EU522093
T-14[a]	F[2]: TAG GCT CAG GGA TAT GAT AGC R: CTC CAG CGA CAG TTG CAA CAG	$(TGC)_8$	0.10	57	2	120-139	3	2.4	0.571	0.591	0.024	EU522094
T-15[e]	F[2]: TGG TAA ATA AGG GCT GCA TGC R: CAG TTT CCT TAC TTT GTC TGT C	$(AC)_{15}$	0.60	55	2	157-309	17	9.9	0.629	0.912	0.118	EU522103
T-17[a]	F[3]: AAC AGT ATT ATG GAT GCA GAC R: GAC ACA AAA GGT ACC ATT CCC	$(TGC)_7$	0.40	57	2	118-136	4	1.5	0.229	0.339	0.049	EU522095
T-26[b]	F[3]: CAG TGA TTT TTG CTA CCA AGG R: GCA AAA CAG TAT TAT GGA TGC	$(GCA)_7$	0.25	57	2	155-176	15	5.9	0.600	0.842	0.014	EU522096
T-27[e]	F[2]: TTC TAG CCC AAC CCA TGT AGC R: GTG GTT ATA AGG AAG TCA TGC	$(TGC)_8$	0.40	55	2	140	4	1.4	0.257	0.303	-	EU522109
T-31[c]	F[1]: GGG ACC ACT CAT GGA ACT AAG R: GGG ATA GAA TTG GGA ATG TAT G	$(AC)_{18}$	0.40	57	2	127-271	17	9.2	0.857	0.905	0.005	EU522104
T-39[d]	F[1]: AAG CAG GGA GTT GCA AAT CAC R: ATC TGG CCT TTG GTC TTT CAG	$(CA)_{36}$	0.20	59	2.5	105-201	18	9.8	0.588	0.911	0.129	EU522105
T-41[e]	F[3]: TCC CTC ACT TCT AGC TCT ACC R: TCT TCT GTC TGG GTG GGT GTG	$(AC)_2C(AC)_{15}\ (ACCC)_3$	0.75	55	2	125-207	11	3.6	0.606	0.734	0.079	EU522106
T-42[a]	F[1]: CCA AAC TTG AAC ACT GCT GTG R: GGA CTC CCA GAT TAT GGT CTC	$(ACC)_8$	0.15	57	2	155-164	2	1.2	0.171	0.205	0.022	EU522097
T-44[b]	F[2]: AAG GCA GTT GAG AAC CAG GTG R: GTA GAT GCC ACC CAT GTT GTC	$(AGC)_7$	0.20	57	2	133-145	4	3.7	0.743	0.740	0.001	EU522098
T-47[d]	F[3]: CAA TAC TAG TCT GCT GTC ACC R: CTA AGT TAC CAA TGC CTC C	$(CA)_{12}$	0.3	59	2.5	118-230	5	3.1	0.647	0.691	0.008	EU522107
T-58[b]	F[1]: TCC TGA AAG GGT GGG CAA AGG R: CTA GAT GAT TCT CAG TCT TTC	$(CAC)_7$	0.25	57	2	154-163	3	1.1	0.114	0.111	0.000	EU522101
T-67[d]	F[2]: TAC CCT TTA GAC TGA GGC AGG R: AGG AAG ATG AAT CAG GGT GAG	$(CA)_{27}$	0.25	59	2.5	106-184	18	10.3	0.676	0.916	0.098	EU522108
T-80[a]	F[3]: CTC ACC TGC AGC CTC TTT CTC R: AGG ACC TTT CAG GAC CCT CAC	$(TGC)_7$	0.30	57	2	144-168	3	2.1	0.457	0.527	0.027	EU522099
T-87[a]	F[1]: CAG CAC TGA TCT GCA AGT ACC R: GCT ACA CCA GTT TCA CTC TGC	$(TGC)_9$	0.30	57	2	124-154	3	2.4	0.600	0.593	0.006	EU522100

([a-e]Multiplex; [1-3]primer labelled with WellRed Dye [1]D4, [2]D3, [3]D2; N_A, number of alleles; N_E, effective number of alleles; H_o, observed heterozygosity; H_E, expected heterozygosity)

Table 5.2 Cross-species amplification of *Chelodina rugosa* microsatellite primers for eight congeners.

	C. burrungandjii			*C. canni*			*C. expansa*			*C .longicollis*		
Locus	Success	No. Alleles	Size Range	Success[a]	No. Alleles	Size Range	Success	No. Alleles	Size Range	Success	No. Alleles	Size Range
T-11	5/5	7	147-181	5/5	5	137-173	3/5	4	163-276	5/5	6	141-159
T-12	5/5	3	157-163	3/5	4	151-163	2/5	2	157-160	2/5	2	160-163
T-14	5/5	3	120-126	4/5	2	117-126	2/5	1	129	2/5	1	120
T-15	5/5	7	165-203	5/5	2	171-173	5/5	4	163-181	5/5	1	171
T-17	5/5	1	118	1/5	1	118	2/5	2	130-133	2/5	2	118-124
T-26	4/5	3	139-158	4/5	2	158-164	3/5	2	158-161	3/5	1	158
T-27	4/5	2	140-157	4/5	2	157-166	0/5	-	-	0/5	-	-
T-31	5/5	7	110-217	5/5	6	133-157	3/5	4	144-306	5/5	7	138-186
T-39	5/5	7	91-195	3/5	3	93-103	0/5	-	-	0/5	-	-
T-41	5/5	3	131-143	4/5	2	135-137	2/5	1	139	4/5	5	133-143
T-42	5/5	3	152-158	4/5	2	152-167	2/5	2	155-158	2/5	1	152
T-44	5/5	3	133-139	4/5	2	136-139	2/5	2	136-145	3/5	1	142
T-47	5/5	2	112-120	3/5	2	118-120	0/5	-	-	0/5	-	-
T-58	5/5	2	157-160	4/5	2	157-166	2/5	2	169-172	3/5	4	157-166
T-67	5/5	7	112-206	2/5	2	94-142	0/5	-	-	0/5	-	-
T-80	3/5	1	147	2/5	1	144	1/5	1	150	2/5	1	144
T-87	5/5	2	136-145	4/5	4	136-148	3/5	1	136	2/5	2	136-145

[a]Success' is the total number of individuals that successfully amplified over the total number of individuals tested.

Table 5.2 (continued) Cross-species amplification of *Chelodina rugosa* microsatellite primers for eight congeners.

	C. mccordi			*C. oblonga*			*C. parkeri*			*C. pritchardi*		
Locus	Success	No. Alleles	Size Range	Success[a]	No. Alleles	Size Range	Success	No. Alleles	Size Range	Success	No. Alleles	Size Range
T-11	5/5	6	135-153	5/5	9	127-255	5/5	3	119-137	5/5	1	143
T-12	2/5	1	157	2/5	2	151-160	3/5	1	160	3/5	2	151-160
T-14	3/5	1	126	4/5	1	120	5/5	1	117	5/5	2	123-144
T-15	5/5	2	163-173	5/5	2	179-181	5/5	1	177	5/5	1	171
T-17	0/5	-	-	4/5	2	133-139	5/5	1	130	0/5	-	-
T-26	5/5	2	146-158	4/5	1	158	4/5	2	158-161	2/5	1	161
T-27	5/5	1	137	5/5	2	137-140	5/5	1	140	5/5	1	137
T-31	4/5	3	132-138	5/5	3	84-88	5/5	6	120-182	5/5	1	136
T-39	5/5	1	93	5/5	8	143-227	5/5	4	137-145	5/5	1	97
T-41	2/5	2	123-131	5/5	3	101-107	2/5	2	155-157	5/5	1	131
T-42	3/5	2	152-155	0/5	-	-	2/5	1	164	5/5	1	152
T-44	5/5	3	136-151	4/5	3	136-142	5/5	1	139	3/5	1	136
T-47	5/5	3	131-137	2/5	2	109-117	0/5	-	-	5/5	1	147
T-58	5/5	2	148-166	4/5	3	166-175	4/5	1	166	2/5	1	166
T-67	0/5	-	-	0/5	-	-	5/5	5	148-196	5/5	3	126-152
T-80	3/5	1	144	4/5	1	144	4/5	1	144	4/5	1	144
T-87	0/5	-	-	4/5	1	145	5/5	2	136-142	4/5	1	145

[a]Success' is the total number of individuals that successfully amplified over the total number of individuals tested.

dinucleotides and trinuceotides, respectively) suggesting that both types of markers will be informative genetic markers.

Primers were tested on five individuals of eight additional species: *Chelodina burrungandjii* (4 populations), *C. canni* (2 populations), *C. expansa* (2 populations), *C. longicollis* (4 populations), *C. mccordi* (1 population), *C. oblonga* (1 population), *C. parkeri* (1 population) and *C. pritchardi* (1 population). The details of the cross-species amplification of the primers are shown in Table 5.2. The rate of amplification success varied across these eight species: 98% in *Chelodina burrungandjii*, 72% in *C. canni*, 38% in *C. expansa*, 58% in *C. longicollis*, 67% in *C. mccordi*, 73% in *C. oblonga*, 81% in *C. parkeri*, and 68% in *C. pritchardi*. Tests of the transferability of the primers suggest that they are (i) highly suitable for *C. burrungandjii* and *C. canni* (15/17 loci polymorphic), (ii) have limited suitability for *C. oblonga* (11/17 loci polymorphic), *C. mccordi* and *C. expansa* (9/17 loci polymorphic), *C. longicollis* and *C. parkeri* (7/17 loci polymorphic), and (iii) are not suitable for *C. pritchardi* (3/17 loci polymorphic) for population genetic, gene flow and hybridisation studies.

In conclusion, these new microsatellite loci will contribute to the genetic monitoring of trade in *Chelodina rugosa*. They will also be used for studies of mating systems, population genetics, gene flow and hybridization in the genus *Chelodina*.

Chapter 6 - Phylogeography of the Australasian freshwater turtle *Chelodina rugosa* and its hybridisation and genetic introgression with two sympatric species *C. burrungandjii* and *C. canni*.

Picture: *Chelodina burrungandjii*.: a species that forms hybrids with *Chelodina rugosa*. Photo by Erika Alacs.

Chapter 6 - Phylogeography of the Australasian freshwater turtle C*helodina rugosa* and its hybridisation and genetic introgression with two sympatric species *C. burrungandjii* and *C. canni*.

Abstract

Phylogeography of the Australasian freshwater turtle *Chelodina rugosa* was investigated using 867 bp of the mitochondrial control and ND4 regions. Two major haplotype lineages were found across the range of *C. rugosa*. The first lineage consisted of haplotypes from the Gulf of Carpentaria, Cape York and southern Papua, and the second lineage was comprised of haplotypes from the Northern Territory. The relationships among mitochondrial haplotypes suggest that the Papua and Australian forms are not distinct taxa and refuted the designation of the Papuan specimens as *Chelodina siebenrocki*. Lake Carpentaria was formed during the Pleistocene (40,000 to 8,000 years ago) and connected river drainages between southern Papua and northern Australia, and thereby facilitated gene flow between *C. rugosa* of eastern Australia and Papua, but not between those of the Northern Territory (western drainages) and Papua. *Chelodina burrungandjii* is sympatric with *C. rugosa* in the Northern Territory and extensive hybridisation between *C. burrungandjii* and *C. rugosa* in the Roper, South Alligator and Cadel Rivers in the Northern Territory was identified by sequencing for two mitochondrial genes (control region and ND4) and genotyping for 17 microsatellite loci. Hybridisation was unidirectional with *C. burrungandjii* males mating with *C. rugosa* females to produce fertile hybrid offspring as evidenced by the formation of a single mitochondrial 'hybrid' haplotype lineage that was closely related to the maternal species, *C. rugosa*. Hybridisation and gene flow between the two sympatric species (introgression) is both historic and contemporary as evidenced by the mitochondrial 'hybrid' haplotype lineage having both *C. rugosa* and *C. burrungandjii* morphotypes and confirmed by the identification of an F2 hybrid in the microsatellite analysis. A *C. rugosa* by *C. canni* hybrid was also identified by the mitochondrial data in combination with morphology, in which characters that were obviously intermediate between the two species were observed. Taxonomic status of *Chelodina* sp. from the Kimberley region was examined but could not be fully resolved based on available molecular (45 allozyme loci, 867 bp of mitochondrial DNA, and 898 bp of nuclear R35 intron) and morphological evidence. Mitochondrial and the nuclear R35 intron (898 bp) gene trees were incongruent in the placement of *Chelodina* sp. (Kimberley)

and *C. canni* as sister taxa. The discrepancy between the mitochondrial and nuclear gene trees was most likely caused by the differential retention of ancestral mitochondrial polymorphisms or ancient introgressive hybridisation between *Chelodina* sp. (Kimberley) and *C. canni*.

Introduction

Phylogeography can provide insights into historical processes (e.g. vicariance events) that have shaped taxonomy, current distributions of taxa and genetic diversity within species (Avise 1998; Templeton 2001). One region of particular interest is northern Australia and New Guinea. Extensive biogeographic, and more recently phylogeographic, studies of this region have revealed the exchange of fauna and flora between southern New Guinea and northern Australia that has occurred as recent as 8,000 years ago (e.g. De Bruyn *et al.* 2004; Kuch *et al.* 2005; McGuigan *et al.* 2000; Rawlings *et al.* 2004; Rawlings and Donnellan 2003; Unmack 2001; Williams *et al.* 2008). There is convincing palaeogeographical evidence for the formation of an extensive Lake Carpentaria that connected drainages of northern Australia and New Guinea during the Pleistocene (Chivas *et al.* 2001; Jones and Torgersen 1988; Reeves *et al.* 2008; Torgersen *et al.* 1983). Lake Carpentaria fluctuated from freshwater to saline between 80,000 and 12,000 years ago when the sea repeatedly breached and withdrew from the Gulf of Carpentaria (Chivas, Garcia et al. 2001). Its largest freshwater phase was between 12,000 to 11,000 years ago, in which it had dimensions approaching 600 x 300 km (180,000 km^2) and a depth of about 15 m (Chivas *et al.* 2001; Reeves *et al.* 2007; Reeves *et al.* 2008). Lake Carpentaria bridge facilitated exchange of terrestrial and aquatic taxa across the Gulf of Carpentaria, Northern Territory, and western Cape York (e.g. De Bruyn *et al.* 2004; Kuch *et al.* 2005; McGuigan *et al.* 2000; Rawlings *et al.* 2004; Rawlings and Donnellan 2003; Unmack 2001; Williams *et al.* 2008). To the east of Lake Carpentaria, a Torres Strait land bridge connected southern New Guinea and Cape York until it was submerged by marine transgression some 8,000 years ago (Nix and Kalma 1972). On the west, the emergence of the Arafura Sill 22,000 years ago that extended from Cape Arnhem in the Northern Territory to southern New Guinea promoted reciprocal exchange of fauna between these regions (Chivas, Garcia et al. 2001).

This study investigates the influence of Lake Carpentaria on the genetic structure of *Chelodina rugosa* and expands on the allozyme study of Georges et al. (2002) with more

extensive sampling and inclusion of sympatric species. *Chelodina rugosa* is a freshwater turtle that inhabits lowland coastal drainages of northern Australia, Gulf of Carpentaria, Cape York and southern New Guinea (Georges, Adams et al. 2002; Georges and Thomson 2002). Specimens from New Guinea have been variously assigned to *C. rugosa* (Georges and Thomson 2002; Goode 1967) or to *C. siebenrocki* (Burbidge, Kirsch et al. 1974; Cogger 1983). There is scant morphological evidence to distinguish them (Rhodin and Mittermeier 1976) and they are not differentiated by phylogenetic analysis of 45 allozyme loci (Georges, Adams et al. 2002; Georges and Thomson 2002). This study uses phylogenetic analysis of two mitochondrial genes (control region and ND4) and the nuclear R35 intron to investigate the taxonomic relationship between *C. rugosa* of southern Papua and northern Australian, and to ascertain whether those of southern Papua should be regarded as a distinct taxon.

Additionally, hybridisation and introgression between *C. rugosa* and its two sympatric species − *C. burrungandjii*, and *C. canni* − was investigated by sequencing two mitochondrial genes, and genotyping 17 hypervariable microsatellite loci. *Chelodina burrungandjii* is considered to be closely related to *C. rugosa* and is described from Arnhem Land in the Northern Territory (Thomson, Kennett et al. 2000). *C. burrungandjii* and *C. rugosa* are occasionally sympatric in Arnhem Land (Thomson, Kennett et al. 2000). Hybridisation between the two species has been documented by the allozyme study of Georges *et al.* (2002), however owing to lack of multiple fixed differences between the two species for 45 allozyme loci they could not establish whether introgression was occurring. In addition, *C. rugosa* x *C. canni* hybrids and backcrossed individuals produced by matings between hybrids and *C. canni* have been identified (Georges, Birrell et al. 1998).

In the Kimberley region of Western Australia another form of *Chelodina* sp. exists that is morphologically most similar to *Chelodina burrungandjii* but was regarded as a distinct taxon based on several diagnosable morphological characters (Thomson, Kennett et al. 2000). We investigated the taxonomic distinction of the Kimberley form from *Chelodina burrungandjii* by sequencing two mitochondrial genes (control region and ND4) and the nuclear R35 intron.

Methods

Sampling and collection of tissue samples

Tissue samples were collected from live turtles caught in modified crab traps or hand captured by snorkelling. Small tissue samples were obtained from the vestigial webbing on the hind foot or from skin along the neck. Blood and liver samples were available from the Turtle Tissue Collection maintained at the University of Canberra (http://aerg.canberra.edu.au/cgi-bin/locations.cgi). Samples were collected from 67 *C. rugosa* from 22 major drainages across the species range in New Guinea and northern Australia (Figure 6.1). A total of 49 samples were collected from the Kimberley form of *Chelodina* representing the seven major drainages across its range (Figure 6.2: localities 1-7). Samples were collected from 13 *C. burrungandjii* from three localities: South Alligator, Cadel and Roper River (Figure 6.2: localities 8-10). A total of ten samples were collected from *C. canni* representing five major drainages (Figure 6.2; localities 10-14).

DNA extraction, mitochondrial DNA amplification and sequencing

Total genomic DNA was extracted using a standard Chelex protocol for skin samples (Walsh, Metzger et al. 1991) or salt extraction protocol for blood samples (Sambrook and Russell 2001). A fragment of approximately 950 bp comprising of the 3′ portion of the mitochondrial ND4 gene and the histidine and serine tRNA genes were amplified for seven samples of each of the species: *C. rugosa*, *C. burrungandjii*, and *C. canni*. The ND4 fragment was amplified in a 25 µl polymerase chain reaction (PCR) containing 20-100 ng template DNA, 0.25 µM of primers ND4F 5′ -CACCTATGACTAC CAAAAGCTCATGTAGAAGC-3′ and LeuR 5′ -CATTACTTTTACTTGGATTTGCACC A-3′ (Arevalo, Davis et al. 1994), 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 1 × PCR buffer, and 0.5 U *Taq* DNA polymerase (Bioline Red *Taq*). The PCR cycling conditions were as follows: 94 ^{o}C for 5 min, followed by 35 cycles of 94 ^{o}C for 45s, 55 ^{o}C for 45s, 72 ^{o}C for 60s, and a final extension of 72 ^{o}C for 6 min. All PCR reactions were run on an Eppendorf Mastercycler 5333 version 2.30 thermal cycler. Polymerase chain reaction products were purified using a standard polyethylene glycol (PEG) procedure (Sambrook and Russell 2001) and sequenced using an ABI automated sequencer at the facility of Macrogen in Seoul, Korea.The 21 ND4 sequences were aligned by eye and an internal primer (ND4intR) was developed that will reliably amplify a fragment of approximately 600 bp for sequencing.

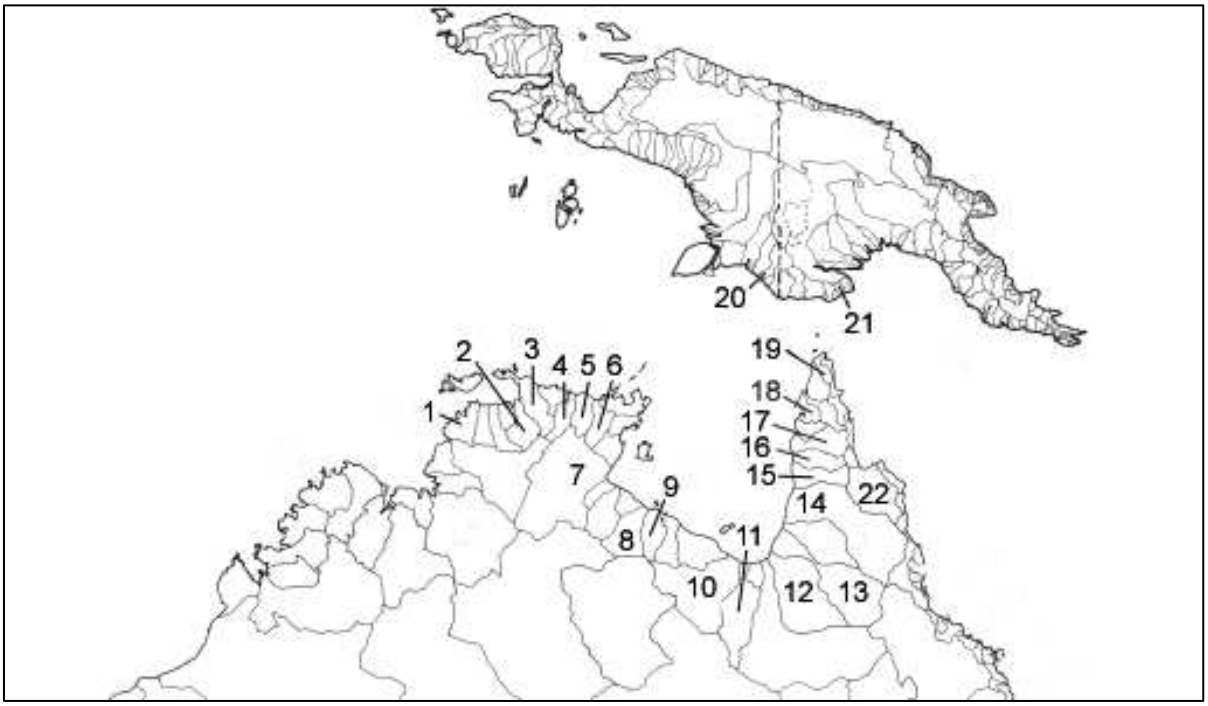

Figure 6.1 Australian, Papua and West Papua drainage basins showing the 22 basins from which *Chelodina rugosa* samples were collected. Major drainage basins are numbered as follows: **1.** Finnis River, **2.** South Alligator River, **3.** East Alligator River, **4.** Liverpool River, **5.** Cadel River, **6.** Goyder River, **7.** Roper River, **8.** MacArthur River, **9.** Robinson River, **10**. Nicholson River, **11.** Leichardt River, **12.** Norman River, **13.** Gilbert River, **14.** Mitchell River, **15.** Edward River, **16.** Holroyd River, **17.** Archer River, **18.** Wenlock River, **19.** Jardine River, **20.** Merauke River, **21.** Binituri River, and **22.** Normanby River.

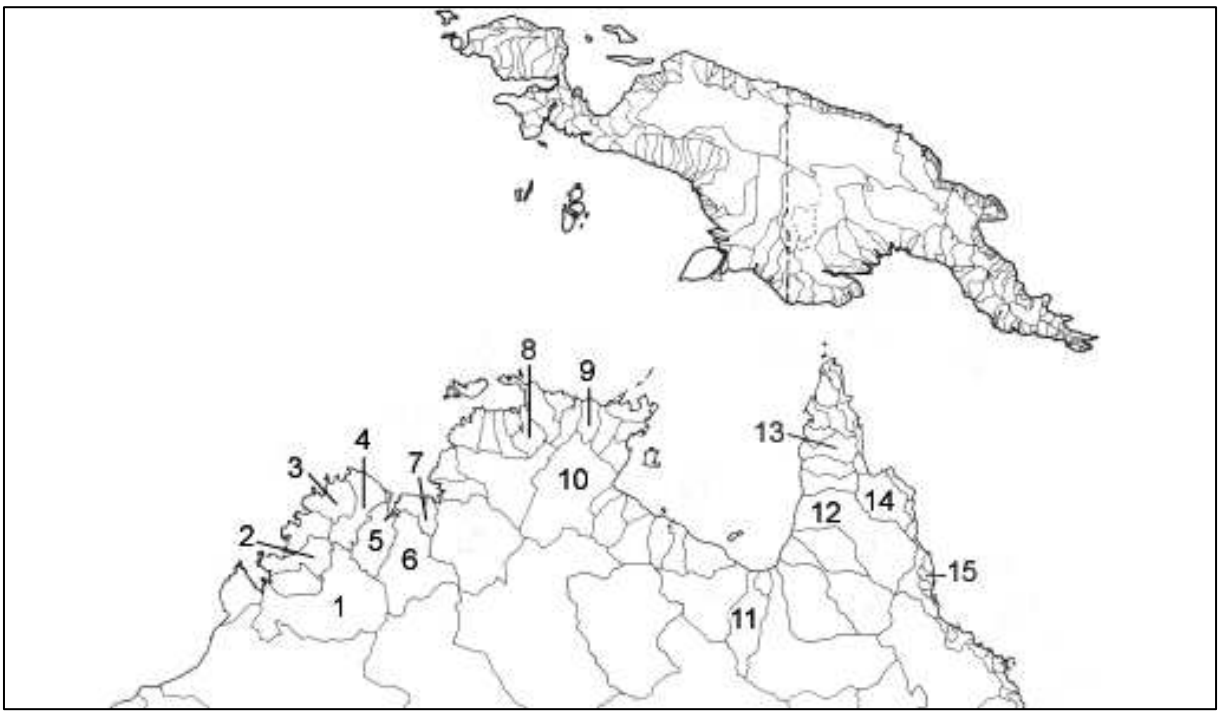

Figure 6.2 Australian, Papua and West Papua drainage basins showing the major river basins from which *Chelodina sp.* (Kimberley), *C. burrungandjii* and *C. canni* samples were collected. Major drainage basins from which *Chelodina sp.* (Kimberley) were collected are numbered as follows: **1.** Fitzroy River, **2.** Isdell River, **3.** King Edward/ Mertens/ Mitchell Rivers, **4.** Drsydale/ Carson Rivers, **5.** Durack/ Pentacost Rivers, **6.** Ord River, and **7.** Keep River. Major drainage basins from which C. *burrungandjii* were collected are numbered as follows: **8.** South Alligator River, **9.** Cadel River, and **10.** Roper River. Major drainage basins from which C. *canni* were collected are numbered as follows: **11.** Leichardt River, **12.** Mitchell River, **13.** Archer River, **14.** Normanby River (*C. canni* x *C. rugosa* hybrid), **15.** Johnstone River.

All samples were subsequently amplified with the primers ND4F 5′ -CACCTATGACTAC CAAAAGCTCATGTAG AAGC-3′ (Arevalo, Davis et al. 1994) and ND4 intR 5′-AGG TGT TCT CGT CTT TG-3′ using the same conditions for PCR reaction, PCR cycling, clean up and sequencing reaction as detailed above.A fragment of approximately 410 bp of the mitochondrial control region (CR) was amplified in a 25 µl polymerase chain reaction (PCR) containing 20-100 ng template DNA, 0.4 µM and TCR500 5′ -CCCTGAAGAAAGAACCGAGGCC-3′ (Engstrom, Shaffer et al. 2004), 0.75 mM each dNTP, 2.5 mM $MgCl_2$, 2.5µl 10 × PCR buffer, 0.6 M betaine, 5 µg BSA and 0.51 U *Taq* DNA polymerase (Bioline Red *Taq*). The PCR cycling conditions were as follows: 94 ^{o}C for 2 min, followed by 35 cycles of 94 ^{o}C for 30 s, 55 ^{o}C for 30 s, 72 ^{o}C for 45 s, and a final extension of 72 ^{o}C for 5 min.

Phylogenetic Analyses of the mitochondrial ND4 and CR.

Sequence data were edited using SEQUENCHER 4.2.2 (Gene Codes Corporation) and then aligned using CLUSTAL X (Thompson, Gibson et al. 1997) in the program Geneious Pro 3.8.2 (Biomatters Ltd). Alignments were verified by eye. Missing data at the ends of the sequences were removed from the ND4 and CR alignments and the alignments were concatenated for further analysis. The combined data set of 867 bp was analysed by maximum parsimony (MP), and maximum likelihood (ML) using PAUP 4.0b10 (Swofford 2000). The MP analyses assumed that character changes were unordered and of equal weight and used a random stepwise sequence addition algorithm with tree-bisection-reconnection (TBR) branch swapping. Modeltest 3.7 (Posada and Crandall 1998) determined that the best fit model of sequence evolution based on Akaike Information Criterion was the Hasegawa, Kishino, Yano 85 (HKY: Hasegawa, Kishino et al. 1985) plus invariable sites (I) and a gamma distribution (G) of rate heterogeneity across variable sites. The estimated parameters under this model, G = 1.5935, I = 0.6244 and Ti/Tv = 6.15, were implemented in the ML analysis conducted in PAUP 4.0b10 (Swofford 2000). The robustness of the consensus trees was evaluated by bootstrap analysis using 1000 and 100 replicates for MP and ML respectively. *Chelodina expansa* and *Chelodina longicollis* were used as outgroup taxa to root the consensus trees. Sequence divergences (*p*) values were estimated between regional phylogroups and between species, and were corrected for within phylogroup diversity (Avise and Walker 1998).

Table 6.1 Locality information for the *Chelodina* samples used in the phylogenetic analysis of the nuclear R35 intron.

Species	Specimen	State and Country	River
Chelodina longicollis	CL72	NSW, Australia	Mary
Chelodina longicollis	AA20521	QLD, Australia	Burnett
Chelodina canni	AA20248	QLD, Australia	Mitchell
Chelodina pritchardi	497	New Guinea	Kemp
Chelodina novaeguinea	456	New Guinea	Merauke
Chelodina reimanni	491	New Guinea	Merauke
Chelodina oblonga	398	WA, Australia	Swan
Chelodina burrungandjii	AL018	NT, Australia	Roper
Chelodina sp. (Kimberley)	N1721	WA, Australia	Durack
Chelodina sp. (Kimberley)	N1208	WA, Australia	Drysdale
Chelodina sp. (Kimberley)	N624	WA, Australia	Isdell
Chelodina rugosa	G85	QLD, Australia	Holroyd
Chelodina rugosa	478	West Papua	Binituri
Chelodina rugosa	AA20078	NT, Australia	Roper
Chelodina rugosa	AA20002	NT, Australia	East Alligator
Chelodina rugosa	Y007	NT, Australia	Robinson
Chelodina expansa	CE107	NSW, Australia	Murray

Confirmation of hybridisation and introgression using the nuclear R35 phylogeny and microsatellites.

Details of the PCR amplification and sequencing conditions for the nuclear R35 intron gene have been described in detail in the methods section of Chapter 8. A portion of this phylogeny is presented here to illustrate the relationships between species from the *Chelodina* genus for a nuclear gene tree (Table 6.1). A total of five *C. burrungandjii* and 55 *C. rugosa* individuals were genotyped for 17 microsatellite loci. *C. burrungandjii* samples represented each three major drainages: South Alligator River (one sample), Cadel River (three samples), and Roper River (one samples).

Samples of *C. rugosa* were from the Cadel River (46 samples from 10 localities), Roper River (two samples) and East Alligator River (seven samples). The amplification and scoring of the microsatellite loci: T-11, T-12, T-14, T-15, T-17, T-26, T-27, T-31, T-39, T-41, T-42, T-44, T-47, T-58, T-67, T-80, T-87 were carried out according to the protocols described in Chapter 5. The microsatellite data were analysed with the program NewHybrids version 1.1 beta (Anderson and Thompson 2002) to identify F1 hybrids, F2 hybrids and backcrossed individuals resulting from matings between *C. rugosa* and *C. burrungandjii.* NewHybrids uses a bayesian based clustering model with Markov Chain Monte Carlo simulations to detect the posterior probability that individuals belong to each of the hybrid classes. Jeffery priors were used in the NewHybrids analyses and run for 1,000,000 iterations with a burning-in of 100,000 iterations.

Results

The final alignment consisted of 867 bp, including 399 bp of the mitochondrial control region and 468 bp of the ND4 region. In total, 224 sites were variable, and 204 of these were parsimony-informative. Seven binary-coded indels were present within the partial sequences of the control region. There were 41 haplotypes observed in *C. rugosa* samples, two haplotypes in *C. burrungandjii*, 16 haplotypes in *Chelodina sp.* (Kimberley), and seven haplotypes in *C. canni*. Maximum parsimony and maximum likelihood analyses produced the same tree topology (Figure 6.3).

Strong bootstrap support was obtained for two major lineages of *C. rugosa* with 2.4% sequence divergence. One haplotype lineage was comprised of 36 different haplotypes observed in 50 individuals from the Gulf of Carpentaria and Cape York

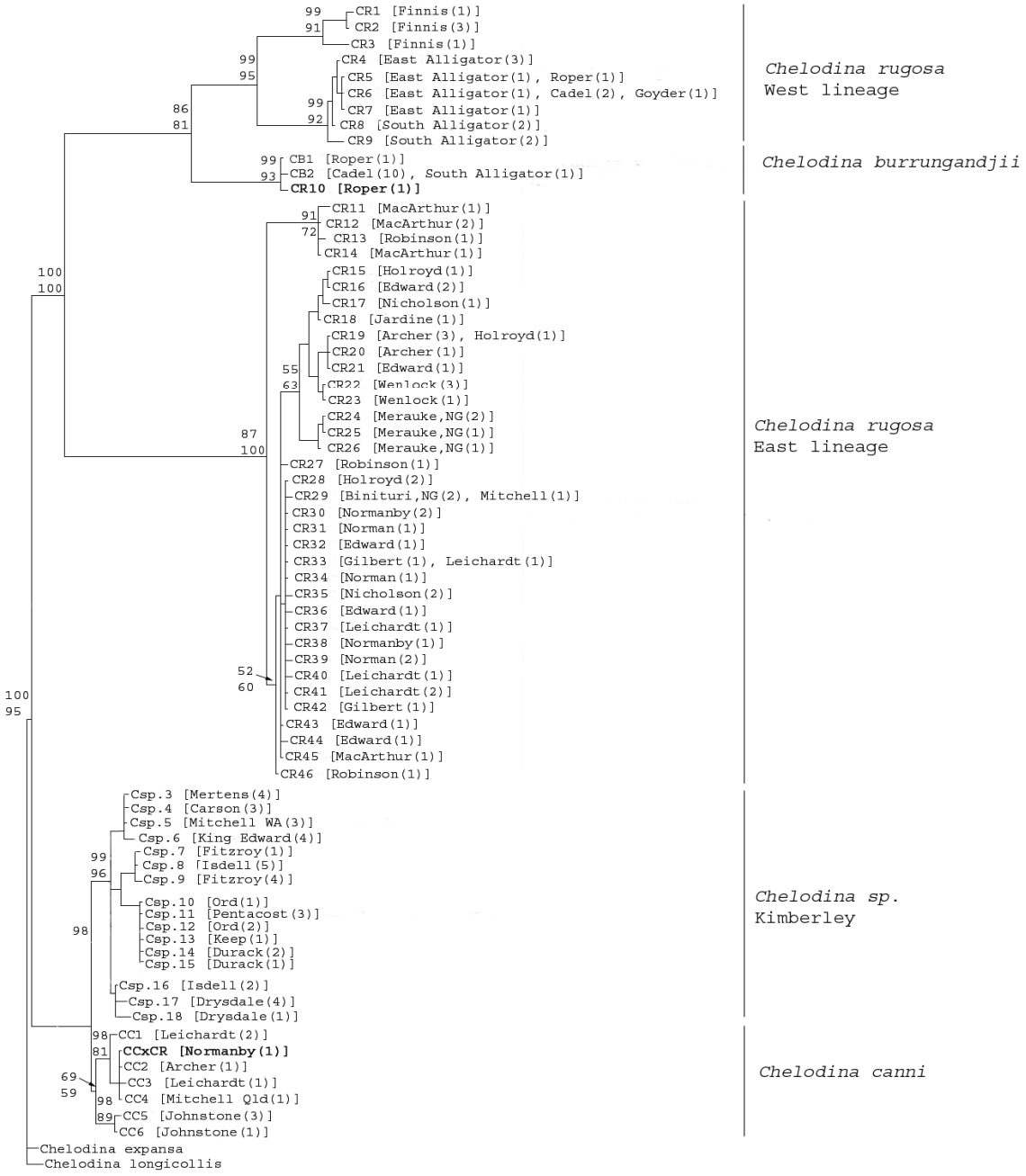

Figure 6.3 Phylogenetic analysis of *Chelodina rugosa*, *C. burrungandjii*, *Chelodina sp.* (Kimberley) and *C. canni* based on 867 bp from the mitochondrial ND4 and control region. Haplotypes are labelled as the species based on morphology (i.e. CR = *Chelodina rugosa*, CB = *C. burrungandjii*, Csp. = *Chelodina sp.* (Kimberley), CC = *C. canni*). Following the haplotype name is information of the main river basins in which they were found and the sample size in parentheses for each locality. Numbers at nodes refer to bootstrap values with 1000 and 100 replicates performed for Maximum Parsimony (value above) and Maximum Likelihood (value below) analyses respectively. Haplotypes in bold have a morphology that is characteristic for a different species compared to their haplotype.

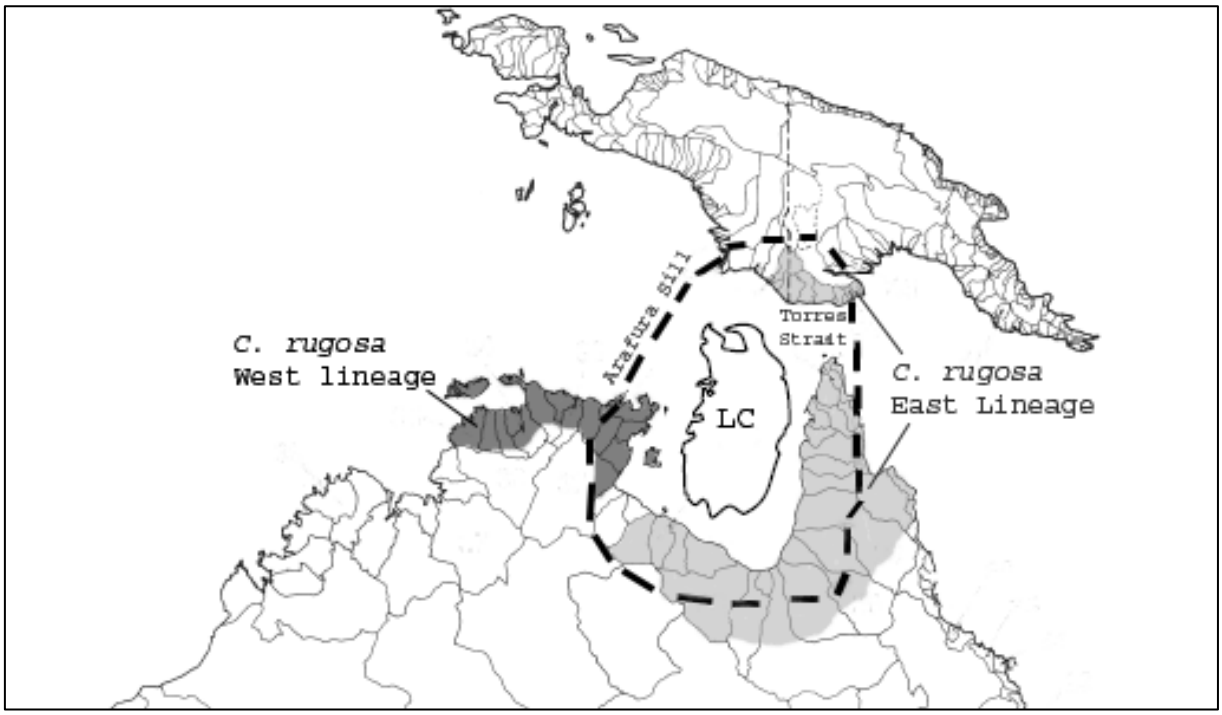

Figure 6.4 Major haplotype lineages for samples from turtles that had *Chelodina rugosa* morphology mapped onto the major drainage basins of Australia, Papua and West Papua. Haplotype lineages were determined by Maximum Parsimony and Maximum Likelihood analyses of 867 bp from the mitochondrial ND4 and control region. The hypothesized position of Lake Carpentaria (LC) and its drainage basin are shown (dashed line) (adapted from Torgersen *et al.* 1985).

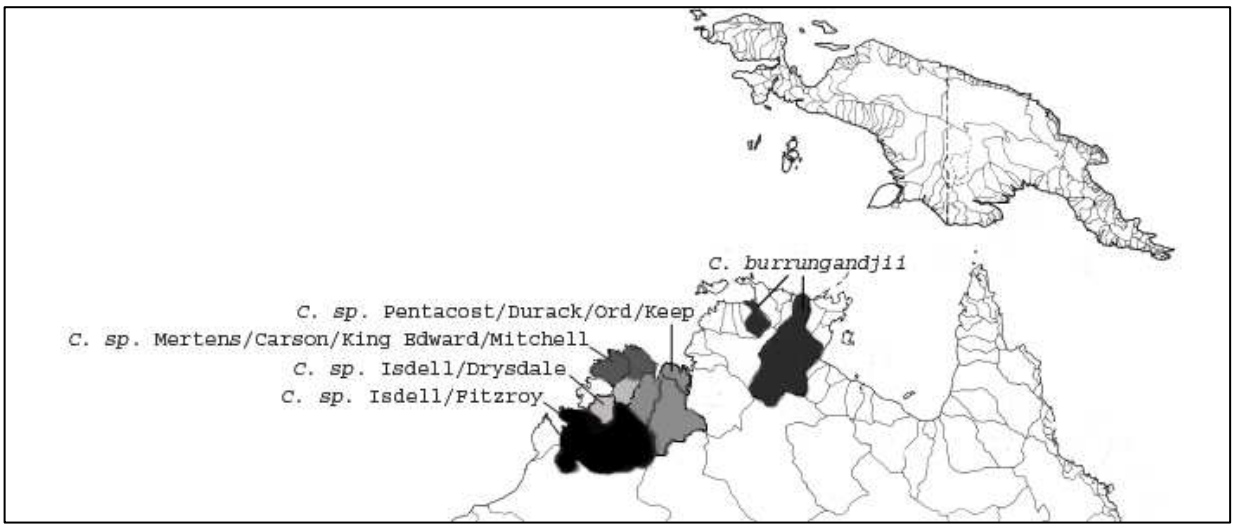

Figure 6.5 Major haplotype lineages for samples from turtles that had the morphology of *Chelodina sp.* (*C. sp.*) from the Kimberley region and *C. burrungandjii* respectively, mapped onto the major drainage basins of Australia. Haplotype lineages were determined by Maximum Parsimony and Maximum Likelihood analyses of 867 bp from the mitochondrial ND4 and control region. *C. burrungandjii* haplotypes occur in the South Alligator, Cadel and Roper Rivers.

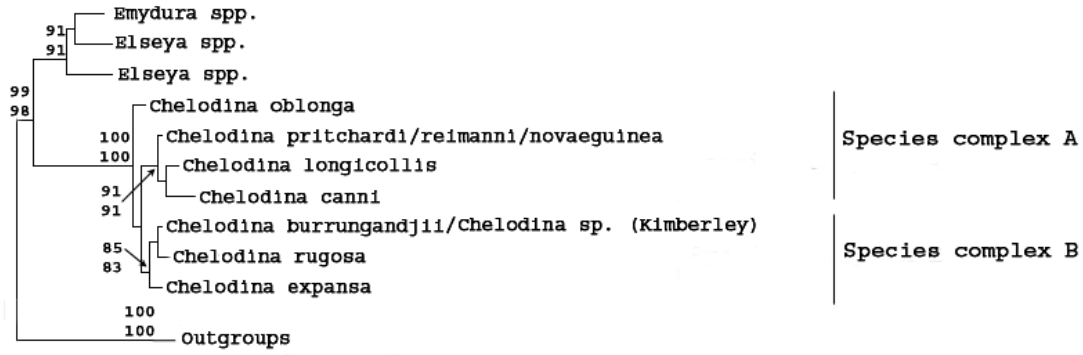

Figure 6.6 Parsimony analysis of selected Australian chelid turtles using 898 bp of the nuclear R35 intron. Node support shown for maximum parsimony with 1000 bootstrap replicates and maximum likelihood with 1000 bootstrap replicates.

drainage basins and individuals from Papua New Guinea (i.e. *C. rugosa*, East lineage; Figure 6.3). The second lineage was comprised of three haplotypes from the Finnis that formed a clade that was closely related to six haplotypes from the South Alligator, East Alligator, Cadel, Goyder and Roper basins (i.e., *C. rugosa* West lineage; Figure 6.3). There was no overlap in the geographic distribution of the East and West haplotype lineages of *C. rugosa* (Figure 6.4). *Chelodina sp.* (Kimberley) mt haplotypes formed a major haplotype lineage that was 3.85% divergent from *C. rugosa* (Figure 6.3). In this major haplotype lineage, there were four minor clades that were 1 to 1.7% divergent in sequence (Figure 6.5).

A clade was formed that contained 12 samples taken from turtles that had *C. burrungandjii* morphotypes and one that had the *C. rugosa* morphotype (i.e. *C. burrungandjii* and *C. rugosa* lineage; Figure 6.3). Of the 12 samples that had *C. burrungandjii* morphotypes, 10 were from the Cadel River, one from the South Alligator River, and one from the Roper River. The haplotype that had the *C. rugosa* morphotype was from the Roper River. The *C. burrungandjii* haplotype lineage was paraphyletic to *C. rugosa* (2.4% sequence divergence) and polyphyletic to the *Chelodina sp.* (Kimberley) (5.7% sequence divergence).

A hybrid origin of the *C. burrungandjii* and *C. rugosa* mt haplotype lineage was supported by microsatellite data. Five samples that belonged to this haplotype lineage (Figure 6.3) and had *C. burrungandjii* morphotypes were analysed for 17 microsatellite loci (Table 6.2). One sample from the Roper basin had high posterior probability (84.2%) of being an F2 hybrid involving *C. burrungandjii* and *C. rugosa.* Two other individuals with C. *burrungandjii* morphotypes from the Cadel basin had weak signatures of backcrossing with 24.3% and 22.0% posterior probabilities of being a backcrossed *C. burrungandjii* (i.e. hybrid x *C. burrungandjii*) and 71.1% and 75.7% of being 'pure' *C. burrungandjii*, respectively (Table 6.2). The two remaining samples that had *C. burrungandjii* morphology had high posterior probabilities of being pure *C. burrungandjii* (>97%; Table 6.2). All the other 56 samples had very high posterior probabilities' of being a pure *C. rugosa* (>97%; data only shown for seven samples in Table 6.2). Additionally a hybrid that was identified in the field by obvious morphological characters that were intermediate between *C. rugosa* and *C. canni*, clustered within the *C. canni* haplotype lineage indicating

Table 6.2 Probabilities of samples being a 'pure' species, hybrids or backcrossed individuals based on NewHybrid version 1.1 beta (Anderson and Thompson 2002) analysis for 17 microsatellite loci (Alacs *et al.* 2009).

Morphology of Sample	mtDNA lineage mtDNA haplotype (drainage)	Pure C. rugosa	Pure C. burrungandjii	F1 hybrid	F2 hybrid (F1 hybrid x F1 hybrid)	F1 hybrid x C. rugosa backcross	F1 hybrid x C. burrungandjii backcross
C. burrungandjii	C. burrungandjii CB2 (Cadel)	0	71.13	0	4.52	0	24.35
C. burrungandjii	C. burrungandjii CB2 (Cadel)	0	75.75	0	2.22	0	22.03
C. burrungandjii	C. burrungandjii CB1 (Roper)	0	0	0	84.18	0	1.58
C. burrungandjii	C. burrungandjii CB2 (Cadel)	0	97.94	0	0	0	1.95
C. burrungandjii	C. burrungandjii CB2 (Cadel)	0	99.68	0	0	0	0.30
C. rugosa	C. rugosa – West lineage CR4 (East Alligator)	99.94	0	0	0	0.63	0
C. rugosa	C.rugosa – West lineage CR6 (East Alligator)	99.98	0	0	0	0	0
C. rugosa	C. rugosa – West lineage CR 4 (East Alligator)	97.65	0	0	2.73	0	0
C. rugosa	C. rugosa – East lineage CR16 (Edward)	99.27	0	0	0	0.22	0
C. rugosa	C. rugosa – East lineage CR16 (Edward)	99.98	0	0	0	0.01	0.21
C. rugosa	C. rugosa – East lineage CR14 (MacArthur)	99.97	0	0	0	0	0.1
C. rugosa	C. rugosa – East lineage CR11 (MacArthur)	99.99	0	0	0	0	0

that it has the maternal *C. canni* mitochondrial DNA haplotype (CC x CR Normanby; Figure 6.3).

Taxonomic distinction of *Chelodina sp.* from the Kimberley region was not apparent from the R35 phylogeny in which it shared the same haplotype as *Chelodina burrungandjii* from Arnhem Land (Figure 6.6). However, distinction was supported by the topology of the mitochondrial phylogeny, in which *Chelodina sp.* (Kimberley) formed a major haplotype lineage that was distinct from haplotypes in *C. burrungandjii* from Arnhem Land (Figure 6.3).

C*helodina canni* formed a clade in the mtDNA phylogeny that was reciprocally monophyletic to haplotypes of the Kimberley form *(Chelodina sp.)* and had high bootstrap support (98% for MP, 93% for ML) suggesting that they are sister taxa that are 1.3% divergent in sequence (Figure 6.3). However, this grouping was incongruent with the nuclear locus. In the nuclear R35 phylogenetic analysis, *C. canni* formed a sister taxon with *C. longicollis* and was part of a monophyletic group that included *C. pritchardi*, *C. reimanni* and *C. novaeguineae* (denoted as species complex A). Species complex A was reciprocally monophyletic with species complex B, which was comprised of *C. burrungandjii* (haplotype is shared with *Chelodina sp.* from the Kimberley region), *C. rugosa* and *C. expansa* (Figure 6.6). *C. burrungandjii* and *C. canni* haplotypes differed by seven bases and 3 indels, compared to (i) one transition mutation (G↔A) and one indel between *C. burrungandjii* and *C. rugosa* haplotypes, and (ii) one transversion mutation (A ↔T) and 3 indels between *C. longicollis* and *C. canni* haplotypes.

Discussion

Taxonomic Issues for Chelodina rugosa

In the mitochondrial analysis, there was no support for the taxonomic distinction of *Chelodina siebenrocki* from New Guinea. Instead, this analysis revealed two major haplotype lineages for *C. rugosa*. The west lineage was comprised of haplotypes from lowland regions of the Northern Territory, extending from the East Alligator to Roper River drainage basins (West lineage; Figure 6.4). The east haplotype lineage extended from the MacArthur River in the Gulf of Carpentaria to Cape York of Queensland (the Type locality) and included specimens from southern New Guinea (East lineage; Figure 6.4). Specimens of *C. rugosa* from New Guinea and northern Australia also shared the

same haplotype for the nuclear R35 phylogeny. These results suggest that New Guinean specimens should not be regarded as diagnosable taxon (i.e. *Chelodina siebenrocki*) but as the same species as *C. rugosa* in northern Australia, and supports the allozyme electrophoresis findings of Georges *et al.* (2002).

I am not suggesting that these two distinct mt haplotype clades are separate biological species, but mtDNA evidence has been used as the sole basis for species designations in the past, and my findings may be used by others as a basis for erecting new species. If this occurs, there are some taxonomic issues that need to be considered. The holotype of *Chelodina rugosa* comes from Cape York (Ogilby 1890), and so the populations bearing the second haplotype lineage would clearly be assigned to *Chelodina rugosa*. However, the holotype of *Chelodina oblonga* (Gray 1841) is actually a *Chelodina rugosa* drawn from populations with my first haplotype lineage, so the name *Chelodina oblonga* would apply to these populations should they be regarded as a distinct species, with *Chelodina colliei* applying to what is currently commonly referred to as *Chelodina oblonga* from south western Australia (Thomson 2006). This is a complex taxonomic issue, and I refer the reader to the submission to the International Union for Zoological Nomenclature by Scott Thomson (Thomson 2006).

Phylogeography of Chelodina rugosa

The phylogeographic patterns observed for *Chelodina rugosa* can be explained by the emergence of an extensive land bridge linking northern Australia and New Guinea during the last glacial maxima when sea levels were 53 m lower than present (Chapell 1994). The land bridge extended from Cape York in Northern Queensland to the Arafura Sill (approximately 500 km east of Darwin; Figure 6.4). The emergence of the Arafura Sill may have facilitated gene flow between Arnhem Land in the Northern Territory and New Guinea. However for *C. rugosa* it appears that gene flow between these regions has not occurred in the recent past. In the Gulf region, Lake Carpentaria fluctuated from freshwater to brackish between 21,000 and 12,000 years ago. It reached its maximum size approximately 12,000 to 11,000 years ago (Couapel *et al.* 2007; Jones and Torgersen 1988; Reeves *et al.* 2007; Reeves *et al.* 2008) with the mighty Fly River of New Guinea flowing into it before being diverted to the east (Torgersen, Jones et al. 1985; Torgersen, Luly et al. 1988). Pollen studies indicate that the vegetation at the fringes of the lake was similar to the open savannah in the Gulf of Carpentaria today (Prebble, Sim et al. 2005). For *C.*

rugosa it appears that Lake Carpentaria facilitated gene flow between populations of the Gulf of Carpentaria, and between Cape York and Southern New Guinea via the Torres Strait land bridge. Exchange of fauna via the Torres Strait land bridge during the Pleistocene is supported by phylogenetic and phylogeographic analyses of several taxa, including freshwater prawns (De Bruyn, Wilson et al. 2004), grass finches (Jennings and Edwards 2005), freshwater melanotaeniid fishes (McGuigan, Zhu et al. 2000), tiger prawns (Ward, Ovenden et al. 2006), and brown snakes (Williams, O'Shea et al. 2008).

The distribution of haplotypes for *C. rugosa* is similar to that observed for the *Liasis* water pythons, in which the eastern Australia haplotypes are more closely related to those of New Guinea than to Northern Territory haplotypes (Rawlings, Barker et al. 2004). Furthermore, the mtDNA sequence divergence (2.5%) of the *Liasis* pythons between the Northern Territory and Eastern Australia/Papua lineages was similar to that of *C. rugosa* (2.4%). The east-west split of lineages in the Gulf of Carpentaria may be a result of the habitat requirements of *C. rugosa*. Like the *Liasis* python, *C. rugosa* requires swampy freshwater to slightly brackish-water habitats in lowland coastal drainages (Kennett, Christian et al. 1993; Rawlings, Barker et al. 2004). If drainages diverting west from Lake Carpentaria were brackish, this may have restricted gene flow of *C. rugosa* between northern Australia and the Gulf of Carpentaria. In contrast to *C. rugosa* and *Liasis* pythons, there was no east-west split in the phylogeographic analysis of the giant freshwater prawn, *Macrobrachium rosenbergii* (De Bruyn, Wilson et al. 2004). The Gulf of Carpentaria populations were grouped in a single diverse lineage that included haplotypes of the Northern Territory and Cape York (De Bruyn, Wilson et al. 2004). *M. rosenbergii* migrates to estuaries to spawn and the juveniles require brackish-water to survive and develop (Ward, Ovenden et al. 2006). This life history trait may have facilitated gene flow between Northern Territory and Gulf of Carpentaria populations via movement of individuals through brackish habitats that were not favourable for the dispersal of *C. rugosa* and *Liasis* pythons. More comparative phylogeographic studies of freshwater- and brackish-tolerant taxa, as well as terrestrial fauna associated with water courses, are required to investigate this phylogeographic break further. Owing to the paucity of recently dated *Chelodina* fossils that are suitable for the calibration of the molecular data, the dating of this east-west split of *C. rugosa* lineages was not attempted.

Hybridisation and Genetic Introgression.

Hybridisation of the two sister species *C. burrungandjii* and *C. rugosa* is evident from allozyme studies (Georges, Adams et al. 2002), mitochondrial phylogeography (this study), and microsatellite data (this study). An F1 hybrid from the Katherine River in the Northern Territory was documented by the allozyme study of Georges *et al.* (2002). Our study found further evidence of hybridisation between the two species in the South Alligator, Cadel, and Roper Rivers. Mitochondrial haplotypes from the South Alligator, Cadel, and Roper Rivers that had *C. burrungandjii* and *C. rugosa* morphotypes formed a single distinct haplotype lineage (i.e. *C. burrungandjii* lineage; Figure 6.3). Microsatellite analysis of six samples from this *C. burrungandjii* and *C. rugosa* lineage detected an F2 hybrid involving *C. rugosa* and *C. burrungandjii.* Additionally, two samples had weak signatures of being *C. burrungandjii* backcrosses (a hybrid and *C. burrungandjii* cross) suggesting that the hybrids are fertile and introgression (i.e. gene flow between the two species) has occurred.

The *C. burrungandjii* haplotype lineage was closely related to the *C. rugosa* West lineage and paraphyletic to the *C. rugosa* East lineage (Figure 6.3). This placement of the *C. burrungandjii* haplotype lineage in the mitochondrial gene tree topology is characteristic of unidirectional hybridisation and introgression (e.g. Donnelly *et al.* 2004; McGuire *et al.* 2007; Sang and Zhong 2000; Stein and Uy 2006; Su *et al.* 2006). In this case it appears that *C. burrungandjii* males have mated with females of *C. rugosa* to produce fertile hybrids and subsequent introgression has taken place. It is not clear whether unidirectional hybridisation is caused by behavioural incompatibilities between *C. rugosa* males and *C. burrungandjii* females that prevents their mating or from asymmetric interspecies sterility, in which a cross between a female of *C. rugosa* and a male of *C. burrungandjii* is fertile while the reciprocal is sterile (Wirtz 1999).

Whether the distinct *C. burrungandjii* lineage consisting of *C. burrungandjii* and *C. rugosa* morphotypes should be recognised as a distinct taxon with hybrid origin is contentious. On the one hand, the hybrids formed their own distinct haplotype lineage suggesting that introgression between *C. burrungandjii* and *C. rugosa* has been extensive and, therefore, that the hybrids are on a separate evolutionary trajectory from their parental species and should be regarded as a distinct taxon (Barton 2001; Dowling and Secor 1997). On the other hand, there was no evidence for reproductive isolation between turtles that had haplotypes belonging to the hybrid (i.e. *C. burrungandjii* lineage; Figure 6.3) and *C.*

rugosa lineages respectively. In fact, to the contrary, the *C. burrungandjii* haplotype lineage had both *C. burrungandjii* and *C. rugosa* morphotypes, suggesting that haplotypes had recent hybrid origins (Figure 6.3). Microsatellite analysis identified and F2 hybrids and backcrossed individuals, confirming recent hybrid origins of haplotypes. These results suggest that the haplotype lineage consisting of *C. burrungandjii* and *C. rugosa* morphotypes (Figure 6.3) has been formed by both historic and contemporary gene flow (i.e. introgression) between the two species. Mallet (2008) suggested that speciation is a continuum rather than a dichotomy and the evolution of species is a gradual process from ecological races and biotypes, to hybridising species and eventually to species that no longer cross. *Chelodina burrungandjii* of Arnhem Land can be regarded as an intermediate in this continuum that has not evolved complete reproductive isolation from its sympatric species, *Chelodina rugosa.* Hybridisation and introgression between *C. burrungandjii* and *C. rugosa* has been so widespread in Arnhem Land that hybrid swamping of *C. burrungandjii* may have occurred, although there is the possibility that our sampling size of 12 was insufficient to identify a 'pure' *C. burrungandjii* haplotype that occurs at low frequency. Further sampling from contact zones would aide in clarifying the relationship between *C. burrungandjii* and *C. rugosa*, and to determine the extent of introgression between the two species.

Lack of reproductive isolation appears to be somewhat common for species in the *Chelodina* genus and hybrids exist even between forms that have distant phylogenetic relationships. Hybrids involving *C. rugosa* and *C. canni* were identified from the Gulf of Carpentaria drainages in north eastern Queensland. One individual from the Normanby River had a morphology intermediate of the two species with a shell resembling that of *C. canni* but head and jaw morphology resembling *C. rugosa*, and a *C. canni* mitochondrial haplotype (this study). An F1 hybrid of *C. rugosa* x. *C. canni,* and a backcrossed *C. canni* individual from the Gilbert River were also documented in the allozyme study of Georges et al. (2002). *C. canni* and *C. rugosa* are sympatric species in the Gulf of Carpentaria, Cape York, and eastern Queensland. Additional sampling of contact zones is required to determine the frequency of hybridisation and extent of introgression between the two sympatric species: *C. rugosa* and *C. canni.*

Taxonomic status and phylogeography of Chelodina sp. (Kimberley)

We were unable to resolve the taxonomic status of the *Chelodina* form in the Kimberley region. In the mitochondrial gene tree, *Chelodina sp.* (Kimberley) formed a distinct haplotype lineage (Figure 6.3) and supported the hypothesis proposed by Thomson et al. (2000) that based on diagnosable morphological characters the Kimberley form is a distinct taxon. In contrast, the allozyme study of Georges et al. (2002) and the nuclear R35 intron (this study) found *Chelodina sp.* (Kimberley) to be synonymous with *C. burrungandjii* from Arnhem Land. However the allozyme and nuclear R35 markers may have lacked the resolution to detect genetic differences between *Chelodina sp.* (Kimberley) and *Chelodina burrungandjii*. Closely related species such as *Chelodina reimanni* and *C. pritchardi* were not distinguishable from each other in the nuclear R35 intron sequences (Figure 6.6), and allozymes can be subject to selection (Sunnucks 2000). However, the mitochondrial analysis was also inconclusive because there is the possibility that our sampling was inadequate to detect a 'pure' *Chelodina burrungandjii* mitochondrial haplotype that could occur at low frequency owing to extensive genetic introgression between *Chelodina burrungandjii* and *C. rugosa*. Sequencing of additional samples of *Chelodina burrungandjii* for the mitochondrial genes is needed to ensure that all mitochondrial haplotypes are represented in the gene tree. Phylogenetic analyses for other nuclear loci that have the resolution to delimit species are also required before the taxonomic status of *Chelodina sp.* (Kimberley) can be fully resolved.

Given the palaeogeographical history of the region it is likely that the Kimberley form has been isolated for a long period of time from *Chelodina burrungandjii* in Arnhem Land. The Arnhem Land and Kimberley Plateaus are hypothesised to be ancient areas of endemism that separated topographically by the formation of basins in the Cretaceous (145-65 my ago). The contraction of rainforest and onset of the monsoonal climate during the Oligocene and Miocene (20-25 my ago) is proposed to have led to further isolation of these regions (Ladiges, Ariati *et al.* 2006; Ladiges, Udovicic *et al.* 2003). Biogeographic and phylogenetic breaks between the Kimberley and Arnhem regions have been found for a diverse array of taxa including grass finches (Jennings and Edwards 2005), freshwater fish (Unmack 2001), eucalyptus trees (Ladiges, Udovicic et al. 2003) and king brown snakes (Kuch, Keogh et al. 2005).

Phylogeographic structuring was observed for *Chelodina sp.* in the Kimberley region with four distinct clades: (i) Eastern Kimberley clade comprised of the Durack, Pentacost, Ord and Keep Rivers, (ii) North Central clade comprised of the King, Edward, Mertens, Carson and Mitchell Rivers, (iii) Isdell/Drysdale Rivers, and (iv) Isdell/Fitzroy Rivers. The phylogeographic structuring of *Chelodina sp.* in the Kimberley region is most likely caused by the rugged topography, in which gorges have provided important refuges for turtles during the dry season. During the wet season flood events may facilitate dispersal events, and hence gene flow, between adjacent basins. Additionally, eastern Kimberley specimens had closely related haplotypes lineages that could be caused by an historical vicariance event lending support to the hypothesis that there was a ridge (currently shallowly submerged) that acted as a drainage divide between the east and west Kimberley during periods of low sea levels (Unmack 2001). The formation of the Northern Central clade suggests that there is substantial gene flow between the Mitchell Rivers (that is connected to Mertens River) and the King Edward River (that is connected to Carsons River). Dispersal of turtles between these two rivers would be via overland dispersal and owing to the risks of overland dispersal is likely to be an infrequent event. Infrequent flooding events that promote dispersal for *Chelodina sp.* (Kimberley) are the most likely explanations for gene flow between the upper Isdell and Fitzroy Rivers. Gene flow between the Isdell and Drysdale Rivers that are over 30 km apart with intervening rugged terrain is probably via the Hann River or Charnley River. Samples from these rivers are required to determine the directions of dispersal and gene flow of *Chelodina sp.* (Kimberley). This study is part of a larger research programme that will extensively sample *Chelodina sp.* (Kimberley) and two other species of freshwater turtle across their range in the Kimberley to compare phylogeographic patterns (FitzSimmons, unpublished data). Studies of freshwater macroinverterbrates and fish found no clear biogeograpgic patterns in this region (Kay, Smith et al. 1999; Unmack 2001), however further phylogenetic studies may reveal additional genetic diversity within these species. Comparative phylogenetic studies are required to reveal whether there are phylogeographic breaks that are shared across taxonomic groups in the Kimberley, a biogeographic region that is one of the least studied in Australia.

Discordance between mitochondrial and nuclear gene trees.

The inferred mitochondrial and nuclear trees differ in the relationships between *Chelodina sp.* (Kimberley) and *C. canni*. The mitochondrial gene tree groups *C. canni* as a sister

taxon to *Chelodina sp.* (Kimberley), while the nuclear gene tree places *C. canni* in a different species complex to that of *Chelodina sp.* (Kimberley). The two *Chelodina* species complexes identified by the nuclear R35 phylogenetic analysis are congruent with relationships found with allozyme electrophoresis (Georges, Adams et al. 2002) and morphological analyses (McCord and Thomson 2002). Several hypotheses have been proposed to explain discordance between mitochondrial and nuclear gene trees (Ballard and Whitlock 2004; Funk and Omland 2003; Maddison 1997). Each of these hypotheses is considered in turn, with some scenarios considered to be more likely than others.

Similar gene topologies were obtained for both the control region and ND4, and individuals sequenced fell into equivalent lineages for both regions. Furthermore, liver samples that are rich in mitochondrial DNA did not produce different results compared to tissue samples of the same individual even in highly diluted samples (1:100 dilutions) in which the nuclear DNA would have been diluted to a point that it would not amplify. This suggests that the mitochondrial results are unlikely to be because of nuclear pseudogenes. Other explanations for discrepancies between the nuclear and mitochondrial gene trees include differntial lineage sorting, incomplete lineage sorting and introgressive hybridisation.

Differential sorting among mitochondrial lineages could have occurred if the common ancestor of *Chelodina sp.* (Kimberley) and *C. canni* had multiple divergent mitochondrial lineages and by random the same mtDNA lineages fixed in *C. canni* and *Chelodina sp.* (Kimberley), and different mtDNA lineages fixed in *C. rugosa* and *C. burrungandjii*. Consequently, *C. canni* and *Chelodina sp.* (Kimberley) would cluster most closely because they share the same ancestral mtDNA lineage, even though they are morphologically, and at the majority of their nuclear genome, dissimilar. Hence a different pattern for nuclear loci would be observed, such as that seen for allozymes (Georges, Adams et al. 2002) and the R35 intron (this study), in which *Chelodina sp. (Kimberley)* was a sister taxa to *C. rugosa* and a phylogenetically distant relative to *C. canni*. If this hypothesis were true, we would expect the divergence between the mitochondrial haplotype lineages of *Chelodina sp.* (Kimberley) and *C. canni* (1.4%) to reflect the time since they shared their most recent common ancestor. Applying the molecular rate of 0.25% sequence divergence per million years as estimated for turtles by Avise (1992) and assuming equal rates of mutation across lineages we can estimate that the most recent common ancestor existed about 5.4 million

years ago in the late Miocene/Early Pliocene. The oldest fossil that has been found for the genus *Chelodina* dates back to the Miocene (Gaffney, Archer et al. 1989). Thus, the hypothesis of incongruence between the mitochondrial and nuclear gene trees caused by the retention of different ancestral mitochondrial polymorphisms in *Chelodina sp.* (Kimberley), *C. canni*, *C. rugosa* and *C. burrungandjii* is plausible. Differential sorting of lineages has also been postulated to explain mitochondrial phylogenies for various taxa ranging from beetles (*Chrysolina auricalcea*; Kitamura *et al.* 2008; *Carabus*; Sota *et al.* 2001) to fish (*Hybognathus*; Moyer *et al.* 2009) and monkeys (Ting *et al.* 2008).

An alternative hypothesis is that differences in gene tree topologies are the result of incomplete lineage sorting. The retention of ancient polymorphism owing to incomplete lineage sorting has been observed for many studies (Crandall and Fitzpatrick 1996; Goodacre and Wade 2001; Hille *et al.* 2002; Klein and Payne 1998; Moran and Kornfeild 1993; Rheindt *et al.* 2009).Owing to the smaller effective population size and faster time to fixation of mitochondria, incomplete lineage sorting is more likely to occur for the nuclear R35 lineages than the mitochondrial lineages (McCracken and Sorenson 2005; Morando *et al.* 2004). If incomplete lineage sorting has occurred then this would lead to the conclusion that *C. canni* is a sister taxon to *C. burrungandjii* based on the mitochondrial results. If this hypothesis were true, we would expect these taxa to have shared derived morphological characters and this has not been found from morphological studies (Cann 1998; Georges *et al.* 2002; McCord and Thomson 2002a; Seddon *et al.* 1997; Thomson *et al.* 2000). Furthermore, incomplete lineage sorting typically is observed when speciation has been very recent, however, a *C. canni* fossil in Queensland has been dated to the Early Pilocene indicating that this species is at least three million years old (Thomson and Mackness 1999). Thus, the evidence suggests that incongruence of mitochondrial and nuclear gene trees is unlikely to be the result of incomplete lineage sorting.

Ancient hybridisation and introgression between the two species might also explain the incongruence between the mitochondrial and nuclear trees. Signatures of ancient hybridisation and introgression have been observed in mitochondrial phylogenies for many taxa (Barbanera et al. 2009; Pastorini et al. 2009; Ray et al. 2008; Trigo et al. 2008; van Herwerden et al. 2006; Zinner et al. 2009) and may have led to the evolution of new species (Grant and Grant 2008; Ropquet and Hassanin 2006). *Chelodina sp.* (Kimberley) and *C. canni* currently have disjunct distributions but they may have had sympatric

distributions in the past (McCord and Thomson 2002; Thomson, Kennett *et al.* 2000). Although hybrids have not been documented between these two species it is plausible that they may have existed in the past. Hybridisation and introgression between species in the genus *Chelodina* is not uncommon. F1 hybrids and backcrossed individuals of *C. canni* with *C. rugosa* have been identified (Georges, Adams et al. 2002; this study) suggesting that there are no reproductive isolating mechanisms between even presumably distantly related species. If ancient hybridisation between *C. canni* and *Chelodina sp.* (Kimberley) occurred, it is expected that some nuclear loci would show the mitochondrial toplogy and others the nuclear R35 topology. If, however, hybridisation was unidirectional with backcrossing over a long period of time it is anticipated that nearly all nuclear loci will carry the same topology as the R35 intron. Additional nuclear loci are required to refute or support the hypothesis of ancient introgressive hybridisation.

Chapter 7 - A genetic perspective on sustainable harvesting of a long-lived species: the Australasian snake-necked turtle *Chelodina rugosa* of Arnhem Land in Northern Australia

Picture: An Aboriginal woman harvesting *Chelodina rugosa* for food using traditional methods. Turtles dig down into the mud where they aestivate for the dry season (August/September to December/January or until the ephemeral swamp fills with water again). Aestivating turtles are located by the presence of excavation mounds or breathing holes on the surface. Probes are used to confirm their locality by the dull thud that is heard when their shells are struck underneath the ground. Photo by Erika Alacs.

Chapter 7 - A genetic perspective on sustainable harvesting of a long-lived species: the northern snake-necked turtle, *Chelodina rugosa*, of Arnhem Land in Northern Australia.

Abstract

The impacts of harvesting on the genetic diversity of populations of the freshwater turtle *Chelodina rugosa* were investigated using a regional and landscape genetic approach. Regional levels of genetic diversity were characterised by sampling populations from river basins adjacent to the Blyth-Cadel basin (Mann/Liverpool and East Alligator basins) and from further afield in the Northern Territory (MacArthur basin) and northern Queensland (Edward basin). Genetic diversity within, and gene flow among, populations from the Blyth-Cadel basin of Arnhem Land that are subject to different harvest regimes was investigated. Genetic diversity estimates were similar for harvested, rarely harvested and unharvested populations (Na = 4.08 – 6.07, *He* = 0.52 – 0.64; 12 polymorphic loci) and no genetic signatures of bottlenecks were detected. Regional structuring between the Blyth-Cadel basin and the Northern Queensland populations were moderate to high (θ = 0.040 – 0.589; *Rho* = 0.079 – 0.446), and also between East Alligator and all other populations (θ = 0.060 – 0.528; *Rho* = 0.067 – 0.367). There was significant population genetic structuring within the Blyth-Cadel region; downstream populations formed a metapopulation with the Thompkinson population of the Mann/Liverpool basin. Upstream populations formed a second metapopulation with substantial gene flow with the downstream metapopulation. Retention of genetic diversity in harvested populations and computer simulations suggest that the metapopulation structure may have buffered the impacts of traditional harvesting over millennia. However, exotic pigs were introduced into the region approximately 35 years ago and actively prey on the aestivating turtles. Pigs pose the greatest threat to the survival of *C. rugosa* of the Blyth-Cadel region based on projections that forecast the loss of genetic diversity over the next 200 years. Genetic monitoring of populations is a tool that could be used to ensure that commercial harvesting is not having an adverse effect on metapopulation dynamics and its contribution to the sustainablillity of that harvest. .

Introduction

Overexploitation has contributed to the extinction of some species (Corlett 2007; Schmolcke and Zachos 2005) and the precarious conservation status of many others

(Bennett et al. 2002; Laurance et al. 2006; Price and Gittleman 2007; Trail 2007; Verlecar et al. 2007). For many surviving populations they have suffered substantial loss of genetic diversity that reduces their adaptive potential (Goossens et al. 2006; Hard et al. 2006; Hauser et al. 2002; Hoelzel et al. 1993; Hwang et al. 2004; Whitehouse and Harley 2001). However, if managed appropriately, harvesting can be conducted in a sustainable manner with no detrimental impact on populations (Bawa and Seidler 1998; Muir 2005; Robinson and Bodmer 1999). Subsistence use of wildlife has occurred in some regions for many millennia with no observable ill effects on the persistence of populations (Bolton 1997b; Borghes 2008; Carpaneto et al. 2007; Corlett 2007; Fordham et al. 2008). The impacts of harvesting on the viability of populations will depend on the intensity and frequency of harvesting, and the species' demographic and genetic responses to harvesting pressures (Bolton 1997a).

The vulnerability of a species to overexploitation is influenced by its life-history traits, compensatory responses to increased mortality, dispersal capabilities, and population structure (Allendorf et al. 2008; Coltman 2008; Harris et al. 2002). In regards to life history, the conservation of long-lived species subject to harvest presents particular challenges. Indeed, whether sustainable harvesting can be achieved at all in such species has been questioned, because they are considered to be more prone to overexploitation than short-lived species (Congdon *et al.* 1993a; 1994; Heppell 1998; Winemiller 2005). Life-history traits that are correlated with longevity, such as delayed sexual maturity, can limit the ability of populations to respond to increased mortality from harvesting (Brooks *et al.* 1991; Congdon *et al.* 1993a; 1994; Congdon *et al.* 1993b; Heppell 1998). For example, if the response to elevated adult mortality is an increase in juvenile recruitment, because of the typically long time required to reach maturity there will be a considerable lag before this compensatory response will take effect (Congdon *et al.* 1993a; 1994). Hence, from a demographic perspective, long-lived species are more vulnerable to overexploitation than short-lived species because recovery from declines will be slower, although this can be alleviated by compensatory responses (Clark and Martin 2007; Congdon *et al.* 1993a; 1994; Heppell 1998; Koons *et al.* 2006).

In contrast, from a genetic perspective, longevity can reduce vulnerability by buffering the loss of genetic diversity that often occurs during drastic declines in population size (also called population bottlenecks) such as those frequently experienced by exploited

populations (Charlier et al. 2008; Hailer et al. 2006; Nabata et al. 2004; Pang et al. 2003; Tejedor et al. 2005). Loss of genetic diversity will occur at a slower rate for species with longer generation times compared to those with shorter generation times but it can still be considerable, and once genetic diversity is lost the recovery of the populations to levels of pre-bottleneck diversity is a slower process (Kuo and Janzen 2004; Loveless and Hamrick 1984).

Whether genetic diversity is lost or retained in harvested populations will also be greatly influenced by the population-level structure that is in turn shaped by the topography of the landscape, local patterns of distribution, and the species' dispersal capability. If opportunity for dispersal is plentiful and contemporary gene flow between populations is high, the detrimental effects of harvesting on any one population are lowered because the impacts will be diffused across all populations that constitute the metapopulation (Allendorf et al. 2008; Coltman 2008; McCullough 1996). If, however, gene flow is unequal and a population is harvested intensively it can act as a sink population and thereby reduce the overall effective population size, alter the rates of gene flow within, and adversely affect the functioning of, the entire metapopulation (Coltman 2008; McCullough 1996; Pannell and Charlesworth 2000). An alternative scenario is that the harvested population is genetically isolated from unharvested regions because of a barrier to dispersal or poor dispersal capability of the species. Isolated populations are highly susceptible to loss of genetic diversity because there is no opportunity for genetic rescue whereby novel genetic material is introduced by the exchange of individuals with other populations (Amos and Harwood 1998). Therefore, the degree of connectivity and levels of contemporary gene flow between harvested and unharvested populations needs to be considered when evaluating the impacts of harvesting on the viability of populations (Allendorf *et al.* 2008).

In this study, I used highly variable microsatellite loci to compare the levels of genetic diversity and gene flow between frequently and rarely harvested populations of a long-lived vertebrate: the freshwater turtle *Chelodina rugosa* from the Blyth-Cadel River (Arnhem Land, Northern Australia). *Chelodina rugosa* is common in ephemeral waterholes and rivers of the wet-dry tropics of Northern Australia and southern New Guinea (Cann 1998). In Northern Australia *Chelodina rugosa* has been favoured as source of protein by Aboriginal peoples for millennia and continues to be an important component of the diet for many communities (O'Dea *et al.* 1991). The Arnhem Land region has a

monsoonal climate with a mean annual rainfall of ~1500mm, of which more than 80% falls between December and March, and high inter-annual variability in onset and extent of monsoon (Holland 1986). Turtles are traditionally harvested during the dry season from August/September to December/January when as the ephemeral waterholes dry up the turtles survive desiccation by burying themselves beneath the ground and aestivating (Kennett and Christian 1994). Aestivating turtles are located by the presence of a breathing hole or mound formed when the turtle buried into the mud. Probes in the form of thin tapered wooden or metal stakes are used to penetrate the mud and the dull thud confirms the presence of an aestivating turtle that is then dug up (Fordham *et al.* 2006). Harvesting is opportunistic owing in part to the variability of rainfall in the wet-dry tropics both within and between years which determines whether the ephemeral waterholes completely dry out and the turtles choose to aestivate. In the past 35 years, traditional harvesting has been compromised by the introduction of exotic pigs that uproot the ground and obscure signs used to locate aestivating turtles as well as actively preying on turtles and causing drastic declines in population sizes (Fordham *et al.* 2006). Since 2003, *Chelodina rugosa* has been the subject of a commercial enterprise for the aboriginal community of Maningrida, situated on the Blyth-Cadel River in Arnhem Land, to supply hatchlings for the Australian pet shop industry.

The impact of harvesting on the genetic diversity of populations is not known, and as such impacts of commercial harvesting, if any, on the retention of genetic diversity, and hence the adaptive potential of populations can not be predicted. This study aims to evaluate the susceptibility of *C. rugosa* populations to loss of genetic diversity from harvesting activities. It will take into consideration the influence of gene flow, compensatory responses to increased mortality and the impacts of pig predation on the retention of genetic diversity in populations. This genetic study will complement the previous demographic and modelling studies that have been conducted for the same populations by Fordham (Fordham *et al.* 2006; Fordham *et al.* 2007; 2008) and provide recommendations on strategies to achieve sustainable commercial harvesting of *C. rugosa* in the Maningrida region.

Methods

Study Populations

A total of 311 tissue samples of *Chelodina rugosa* were collected from 2000 to 2005 from seven sites in the Blyth-Cadel basin of Arnhem Land, Northern Territory, Australia (sample size, population abbreviation, and GPS location in parenthesis): Gidadella (GID; *N* = 33; 12° 31' 33" S, 134° 21' E), Imimbar 1 (IM1; *N* = 41; 12° 44' S, 134° 31' 48" E), Imimbar 2 (IM2; *N* = 35; 12° 44' 37" S, 134° 32' E), Garromgarrom (GGM; *N* = 28; 12° 18' 54" S, 134° 30' 23" E), Garoada (GAR; *N* = 14; 12° 40' 11" S, 134° 41' 43" E), Damdam (DAM; *N* = 34; 12° 9' 53" S, 134° 37' 36" E), Day (DAY; *N* = 18; 12° 37' 08" S, 134° 43' 02" E), and Murrybulljuluk (MUR; *N* = 36; 12° 36' 59" S, 134° 51' 42" E). In addition, samples were collected in 2004 from two basins adjacent to the Blyth-Cadel basin: Thompkinson River (TOM;*N* = 32; 12° 12' 49" S, 134° 17' 01" E), and East Alligator River (ALI; *N* = 9; 12° 14' 55" S, 133° 8' 50" E). Northern Queensland samples were represented from two localities collected in 2004: MacArthur River (ART; *N* = 18; 15° 56' 20" S, 136° 12' 30" E), and Edward River (EDW; *N* =13; 14° 50' S, 142° 9' 20" E) (Figure 7.1).

Microsatellite analysis

DNA was extracted from small slithers of skin from the clawless hind toe using a standard salting–out protocol (Dethmers *et al.* 2006). Samples were genotyped using seven dinucleotide loci (T-11, T-15, T-31, T-39, T-41, T-47, T-67) and nine trinucleotide loci (T-12, T-14, T-17, T-26, T-42, T-44, T-58, T-80, T-87). The isolation of the microsatellites, primer design, conditions for multiplex polymerase chain reaction (PCR), and separation of fragments on the Beckman Coulter CEQ 8000 Genetic Analysis System have been described previously in Alacs *et al.* (2009).

Data validation and within population statistics

The presence of genotyping errors, such as scoring of stutter peaks, non-amplification of null alleles and dominance of smaller alleles was assessed with MICRO-CHECKER software (Van Oosterhout *et al.* 2004). Conversions of data file formats for various population genetic software programs was implemented in GeneAlex v5.1 (Peakall and Smouse 2006), Create v1.0 (Coombs *et al.* 2008) and Convert (Glaubitz 2004). Genetic diversity for each locus were assessed by estimating the mean number of alleles (*Na*;

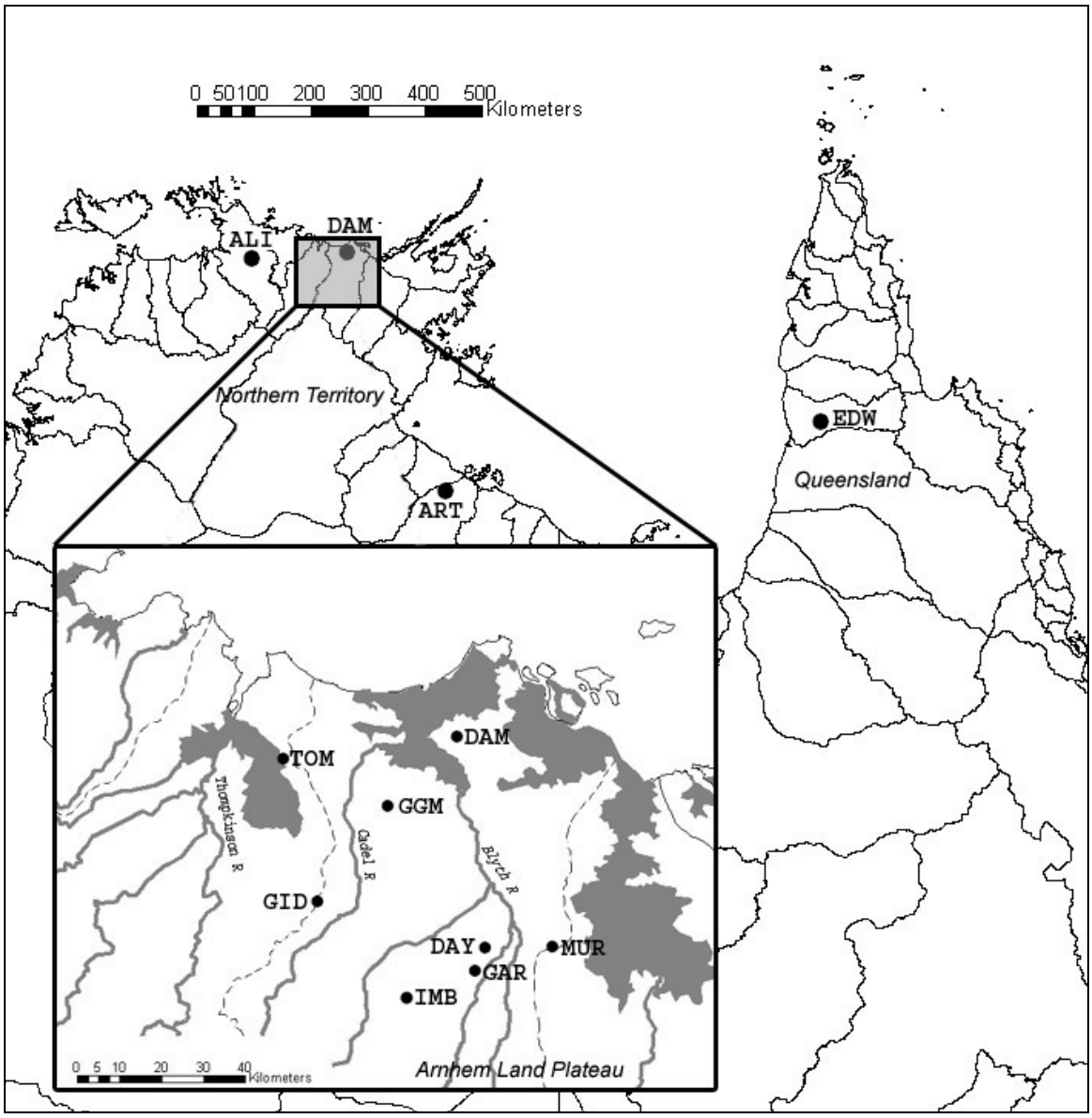

Figure 7.1 Map of the major river basins of northern Australia indicating sampling locations for the population genetic study of *C. rugosa*. Insert shows sampling in the Blyth-Cadel and Mann-Liverpool basin (basin boundaries indicated by dashed lines) of northeast Arnhem Land, Northern Territory. Shaded regions indicate ephemeral floodplains.

(Kimura and Crow 1964) expected heterozygosity (*He*) and observed heterozygosity (*Ho*) using Popgene v1.3 (Yeh and Boyle 1997). GenePop 3.4 (Raymond and Rousset 1995b) was used to test for genotypic linkage disequilibrium between all pairs of loci and to test for deviations from Hardy-Weinberg equilibrium for each loci in each population. Bonferroni corrections (Bonferroni 1936) for multiple comparisons were applied. Overall and within population genetic diversity was estimated by the mean number of alleles (*Na*), allelic richness (*Rs*), observed heterozygosity (*Ho*) expected heterozygosity (*He*). Allelic richness was calculated using Fstat v2.9.3 (Goudet 1995) by the method of El Mousadik and Petit (ElMousadik and Petit 1996) that corrects for variances in the sample sizes of the populations. Animalfarm 1.0 (Landry *et al.* 2002) was used to test the equal contribution of all loci to estimators of genetic distance based on the stepwise mutation model (SMM).

Evidence for recent population bottlenecks or expansions was assessed determining if there were signatures of significant heterozygosity excess or deficiency using the program BOTTLENECK (Piry *et al.* 1999). For these analyses the Wilcoxon sign-rank test was conducted over 1000 iterations using the stepwise mutation model (SMM) of microsatellite evolution and the two phase mutation model (TPM). The chosen TPM consisted of a mixture of 90% stepwise mutations and 10% infinite allele with a 10% variance. Heterozygosity excess can be used to detect severe and recent population bottlenecks but this signature is transient. The M-ratio test statistic of M = *k*/*r*, where k is the number of alleles and *r* is the range of allele sizes was used to detect population bottlenecks that have occurred over a longer period of time (Garza and Williamson 2001: available from http://137.110.142.7/textblock.aspx?Division=FED&id=3298). When rare alleles are lost during a bottleneck, *k* will be reduced to a greater extent than *r*. This signature will persist even if the pre-bottleneck population size was large, the bottleneck occurred over several generations, or the population recovered demographically (Williamson-Natesan 2005).

Population genetic structure

Levels of genetic differentiation among populations were assessed by Weir and Cockerham (Weir and Cockerham 1984) estimator of F_{ST} (denoted θ) and an unbiased estimator of Slatkin's R_{ST} (Slatkin 1995) termed *Rho*, between each pair of populations using Fstat v2.9.3 (Goudet 1995) and RstCalc 2.2 (Goodman 1997), respectively. The F statistics assume an infinite allele model (IAM) of microsatellite mutation, whereas R statistics assume SMM mechanisms and are sensitive to details of mutation process

(Oliveira *et al.* 2006). Isolation-by-distance structuring where genetic distance increases with the geographic distance of populations from each other was tested using Mantel's test in Genepop (Mantel 1967; Raymond and Rousset 1995a; b). Distances were measured by the shortest river and land path, considering that *C. rugosa* is capable of dispersing across land (Fordham *et al.* 2007), between each pair of populations and the significance was assessed by Spearman's rank correlation with 10,000 permutations (Spearman 1904). The genetic distances between populations were calculated using the Cavalli-Sforza-Edwards distance (Cavalli-Sforza and Edwards 1967) and graphically represented in a neighbourhood-joining dendrogram with the robustness of the represented genetic similarities evaluated using 100 bootstrap replicates of the data using the Phylip 3.6.7 computer package (Felsenstein 1989)

Clustering methods were used to identify the number of populations in the Northern Territory, and then to assign individuals to the most probable population of origin. Bayesian clustering methods with an admixture model in which individuals can have mixed ancestry with genotypic contributions from different populations was used to assess population structure in the Northern Territory in the program Structure v2.2 (Pritchard *et al.* 2000). The admixture model was run for K = 1 to 12 clusters. Each run was for one million iterations with a burn in period of 100,000 iterations. The K that best represented the data was chosen by plotting log $\Pr(X \mid K)$ for five runs of each K that is set from one to 12 and then choosing the K at which the plot reaches an asymptote as recommended by (Pritchard *et al.* 2000). The analysis was then run ten times at K = 5 which was the number of clusters that was found to best represent the data. Q-values which are the posterior probabilities of individuals belonging to each of K clusters were plotted.

The Structure analysis was compared to the spatially explicit clustering method of Geneland (Guillot *et al.* 2005) to identify population structure of *Chelodina rugosa* within the Northern Territory region. The number of populations in Geneland was set from a minimum of one to a maximum of 13 for five runs. Each run was conducted for 10,000 iterations with a thinning of 1,000 iterations. The resultant number of populations identified from the above analyses was then used to set the number of populations to a fixed value and this was run 10 times with the same parameters as described above. Running the analyses at least five times is recommended by Guillot *et al.* (2005) to ensure that the Monte-Carlo Markov chains have converged for each run.

Computer simulations

All simulations conducted in Bottlesim v2.6 (Kuo and Janzen 2003) assume negligible gene flow of the population with others and a single harvest (or predation event) of the population. All simulation parameters except population size, and age to maturity remained constant and were set as follows: constant population size, overlap of generations = 100 (i.e. all individuals start with a random age value), dioecy with random mating system, and longevity = 30 years. Simlations assume no selection, migration or mutation (Kuo and Janzen 2003).

A compensatory response of an earlier onset of female maturity was demonstrated in the frequently harvested population of Damdam, in which females typically mature at three years of age compared to six years of age for unharvested populations (Fordham *et al.* 2007). The effect of this compensatory response on the retention of genetic diversity in Damdam was investigated using computer simulations for 200 years with an age of maturity of three and six years, respectively. In addition, simulations with various initial population sizes were run for Damdam to estimate the size of the population that would be required to retain 90% of the current total genetic diversity: an estimate that is also an approximation of the effective population size (Kuo and Janzen 2003; Kuo and Janzen 2004).

To predict the rate of genetic loss under various scenarios, computer simulations were conducted for two frequently harvested populations that have pig predation: Gidadella and Damdam, and for two populations that were infrequently harvested that have low pig abundance: Murrybulljuluk and Imimbar using the program Bottlesim v2.6 (Kuo and Janzen 2003). The age of reproduction was set to five, three, five and six years for Gidadella, Damdam, Murrybullujuluk, and Imimbar respectively based on estimates of Fordham *et al.* (2007). The initial pre-bottleneck size was set to population size estimates for 2004 of Fordham *et al.* (2007) of 125, 147, 179, and 243 turtles for Gidadella, Damdam, Murrybulljuluk, and Imimbar, respectively. These estimates are conservative in that they assume that the effective population size approximates the census population size for 2004. In reality, the effective population size is often only a small fraction of the census population size (Frankham 1995). The census population sizes were used for the simulations rather than estimates of effective population size because demographic studies

suggested that the 2004 population sizes were well below the carrying capacity of the locations and could be lower than the norm due to inter-annual variability in natural mortality (Fordham *et al.* 2007). For each population the loss of genetic diversity over 200 years using 1000 iterations was simulated for several scenarios: (i) 20%, 30% and 40% of the total population harvested, (ii) pig predation based on the mortality rates for each population as estimated by Fordham *et al*. (2006), and (iii) 20%, 30% and 40% of the total population harvested with pig predation. The estimated rates of mortality from pig predation were 48% of the total population in Gidadella and 58% in Damdam (Fordham *et al.* 2006). Murrybullujuluk and Imimbar have very low pig abundance and pig predation is negligible (Fordham *et al.* 2006), hence the scenarios involving pig predation were not simulated for these populations. Harvest intensities are the total percentage of the population removed by traditional or commercial harvesting practices.

Results

Genetic diversity of populations

Genotypes at 17 loci were obtained for 311 *Chelodina rugosa*. Genotypic linkage disequilibrium was found between loci T26 and T17, and with loci T26 and T41 ($P < 0.0001$). Locus T26 was omitted from further analyses and independence between remnant loci was assumed. Locus T27 was monomorphic. The loci T39, T67, and T15 were also omitted from further analyses because they displayed significant deviations from Hardy-Weinberg equilibrium with heterozygote deficits and possible evidence for null alleles detected by MICROCHECKER (Van Oosterhout *et al.* 2004). There were no locus and population comparisons that were significantly out of Hardy-Weinberg equilibrium (HWE) after Bonferroni corrections for multiple comparisons ($\alpha = 0.05$, $p < 0.000347$).

Mean number of alleles ranged from two to four for trinucleotide loci, and nine to 14 for dinucleotide loci (Figure 7.1). Mean number of alleles (*Na*) was similar across populations, ranging from 4.08 to 6.67. Measures of allelic richness, observed heterozygosity and expected heterozygosity were also similar, suggesting the retention of relatively high levels of genetic diversity for all populations (Figure 7.1). There was no evidence of recent population bottlenecks or population expansion in the Wilcoxon sign-rank test under both the SMM and TPM or skewed allelic frequency distributions, nor was there any evidence for a genetic bottleneck revealed by the M ratio statistic (all M values above 1.0, data not shown).

Table 7.1 Measures of genetic diversity among *Chelodina rugosa* populations estimated using 12 microsatellite loci. Abbreviations: *N* = number of individuals genotyped, *Na* = mean number of alleles, *Rs* = allelic richness, H_O = observed heterozygosity, H_E = expected heterozygosity.

	Type of waterhole	*N*	*Na*	*Rs*	H_O	H_E
East Alligator	Annually dry, Harvested	9	4.25	2.97	0.648	0.642
Thompkinson	Annually dry, Harvested	32	6.67	3.01	0.572	0.621
Gidadella	Annually dry, Harvested	33	5.42	2.67	0.528	0.538
Garromgarrom	Annually dry, Unharvested	28	5.25	2.73	0.564	0.576
Imimbar1	Rarely dry, Unharvested	41	6.42	2.87	0.606	0.619
Imimbar2	Rarely dry, Unharvested	35	5.75	2.75	0.526	0.591
Damdam	Annually dry, Harvested	33	6.00	2.83	0.557	0.600
Garoada	Annually dry, Rarely harvested	14	5.00	3.00	0.590	0.626
Day	Annually dry, Rarely harvested	18	4.75	2.80	0.612	0.602
Murrybulljuluk	Annually dry, Rarely harvested	36	5.75	2.99	0.606	0.597
MacArthur	Annually dry, Rarely harvested	18	4.08	2.78	0.489	0.516
Edward	Rarely dry, Unharvested	13	5.00	2.87	0.461	0.579

Population genetic structure

All loci contributed equally to genetic distance estimates based on SMM (ANIMALFARM 1.0 (Landry *et al.* 2002). The overall F_{ST} value calculated across all populations was 0.083 (95% CI 0.058 – 0.116) and the overall *Rho* value was 0.122. Distances between populations ranged up to 240 km between populations of the Northern Territory (including East Alligator) and from 2 km to 120 km in the Arnhem Land region. Tests for isolation by distance were significant when all populations were included in the analysis [(F_{ST}/ F_{ST}-1) vs. log geographic distance; R^2 = 0.57, P < 0.001 based on θ statistics and R^2 = 0.44, P < 0.001 based on *Rho* statistics; Figure 7.2]. When the comparison was limited to populations within the Northern Territory the relationship was not significant (R^2 = 0.24, P >0.05 based on θ statistics and R^2 = 0.22, P >0.05 based on *Rho* statistics; Figure 7.3).

All pairwise θ and *Rho* values between the two Queensland populations (MacArthur and Edward River) and the Northern Territory populations were significant (Table 7.2). Within the Northern Territory, all populations in the Blyth-Cadel basin were significantly different from the East Alligator population (denoted ALI in Table 7.2) based on both θ and *Rho* pairwise tests. The Thompkinson population was significantly different from all populations sampled in the Blyth-Cadel basin except for Garrom for both θ and *Rho* pairwise tests. Garoada was not significantly different from the Thompkinson population for θ pairwise tests but this was not supported by *Rho* pairwise tests (Table 7.2), genetic distance relationships (Figure 7.4) or spatial Bayesian clustering analyses (Figure 7.5). Structure analyses predicted three distinct clusters; (i) Edward River of Queensland, (ii) MacArthur River of Queensland, and (iii) populations of the Northern Territory. No structuring was detected by Structure analyses within the Northern Territory populations, even when the Queensland populations were removed from the analyses (Figure 7.6). Lack of geographic information in the Structure analyses coupled with high rates of dispersal are likely to have resulted in its poor performance compared to Geneland analyses to detect fine scale genetic structuring.

In the Arnhem Land Plateau there was strong support for grouping of the Day, Garoada and Imimbar (both 1 and 2 populations) populations. There was no significant genetic differentiation among these populations based on pairwise θ and *Rho* values (Table 7.2), close genetic distance relationships among them formed a group in the Neighbourhood

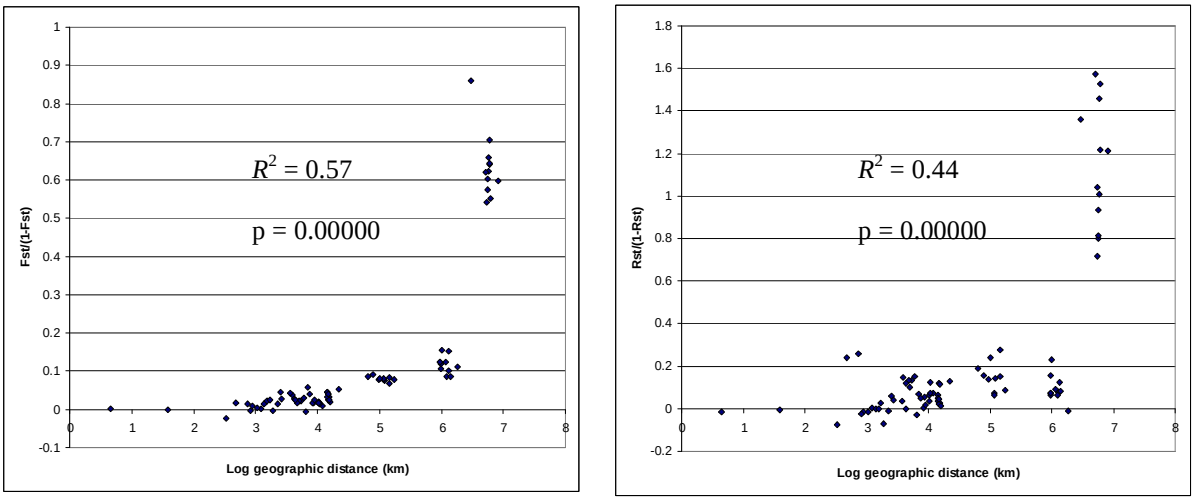

Figure 7.2 Regression of isolation-by-distance of 12 populations of *Chelodina rugosa* based on θ statistics (left) and Rho (right).

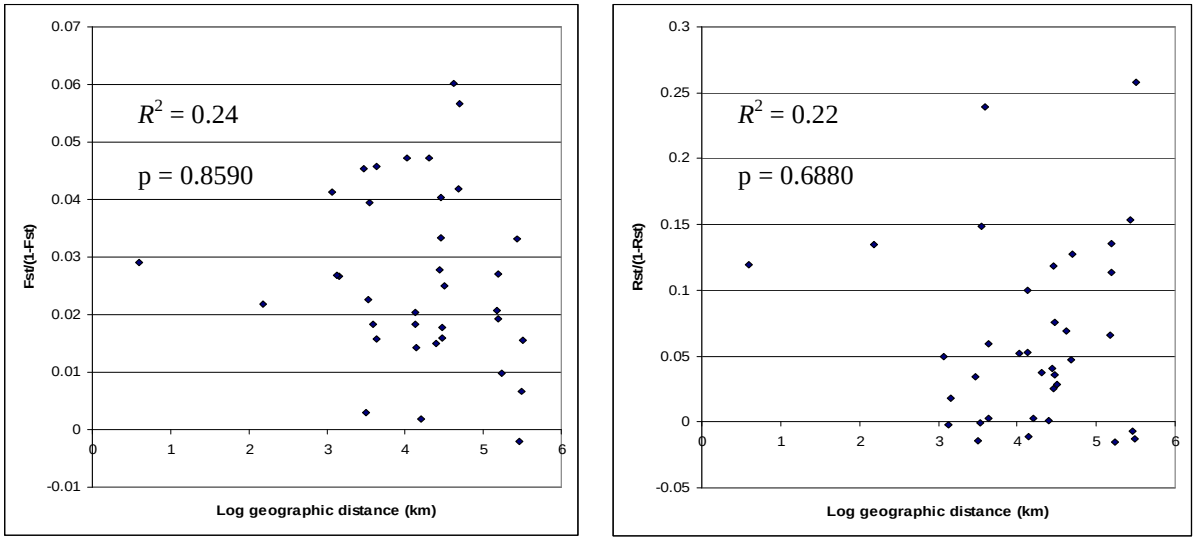

Figure 7.3 Regression of isolation-by-distance for *Chelodina rugosa* populations within the Northern Territory based on θ statistics (left) and Rho (right).

Table 7.2 Pairwise tests of genetic differentiation for 12 Chelodina rugosa populations with θ (upper diagonal) values and Rho averaging over variance components (lower diagonal) based on 12 microsatellite loci. All values in bold are not significant after Bonferroni correction (α = 0.05, $p < 0.00076$).

	ALI	TOM	GID	GGM	IMB 1	IMB 2	DAM	GAR	DAY	MUR	ART	EDW
East Alligator	0	0.082	0.086	0.076	0.078	0.077	0.075	0.067	0.082	0.078	0.098	0.367
Thompkinson	0.119	0	0.043	**0.024**	0.045	0.040	0.026	**0.026**	0.039	0.054	0.080	0.343
Gidadella	0.116	0.041	0	0.014	0.044	0.027	0.015	0.018	0.020	0.017	0.112	0.407
Garromgarrom	0.099	**0.010**	0.009	0	0.057	0.039	**0.002**	0.032	0.021	0.026	0.081	0.380
Imimbar 1	0.061	0.038	0.033	0.031	0	**0.003**	0.045	**0.010**	**0.015**	0.038	0.110	0.367
Imimbar 2	0.069	0.039	0.019	0.023	**0.000**	0	0.032	**0.007**	**0.022**	0.028	0.099	0.386
Damdam	0.108	0.019	**0.007**	**0.004**	0.038	0.018	0	0.020	0.018	0.016	0.091	0.378
Garoada	0.060	0.055	0.039	0.046	**0.003**	**-0.008**	0.032	0	**-0.002**	0.015	0.108	0.342
Day	0.096	0.045	0.024	0.021	**0.015**	**0.016**	0.028	**0.009**	0	0.018	0.131	0.365
Murrybulljuluk	0.101	0.066	0.014	0.019	0.043	0.026	0.031	0.037	**0.019**	0	0.110	0.379
MacArthur	0.071	0.043	0.093	0.040	0.050	0.062	0.069	0.080	0.099	0.112	0	0.446
Edward	0.528	0.562	0.593	0.570	0.559	0.561	0.550	0.541	0.564	0.565	0.590	0

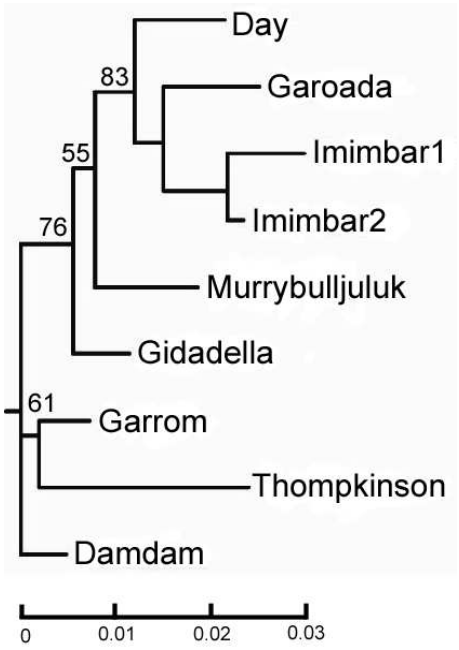

Figure 7.4 An unrooted neighbourhood-joining dendogram of nine *Chelodina rugosa* populations in the Northern Territory, Australia, based on Cavalli-Sforza chord measure (Cavalli-Sforza and Edwards, 1967). Bootstrap values are shown on nodes for 100 replicates.

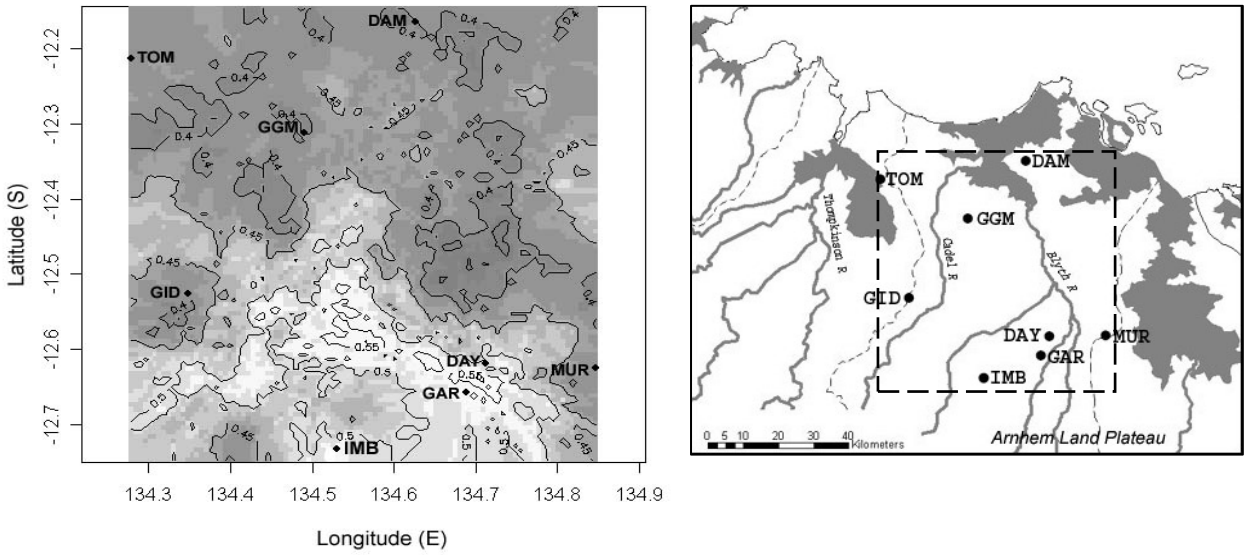

Figure 7.5 Map (on left) showing the posterior probabilities depicted by shading for 271 *C. rugosa* from the Mann/Liverpool and Blyth-Cadel regions of the Arnhem Land Plateau (refer to boxed region of map on right) of belonging to one of the two population clusters identified by Geneland analyses: ☐ cluster 1 (100% probablility) and ■ cluster 2 (100% probability).

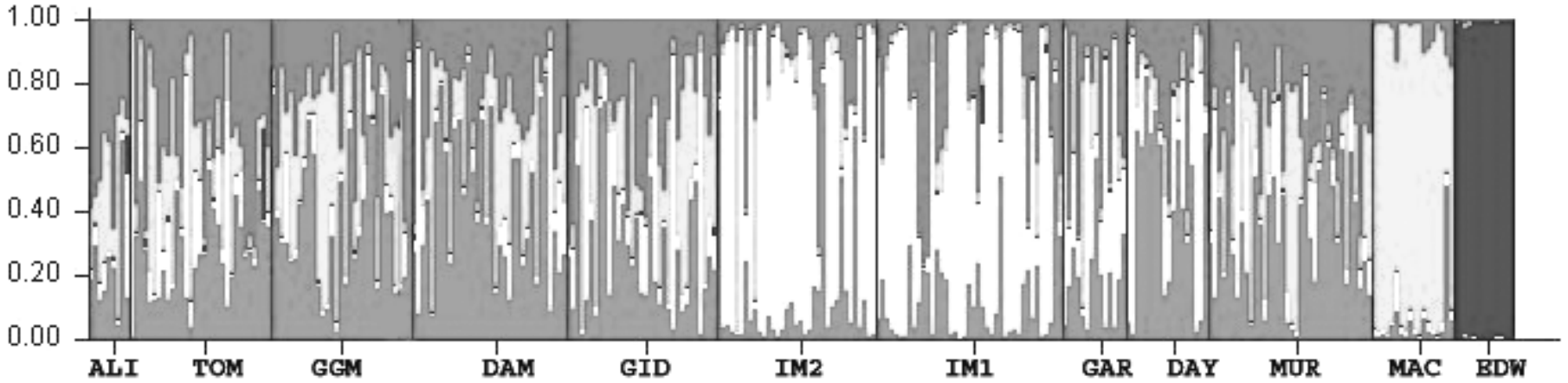

Figure 7.6. A Q-plot for the posterior probability of each individual belonging to five clusters (i.e. populations) that were identified by the admixture model in the Structure analysis, in which: □ is cluster1, □ is cluster 2, □ is cluster 3, ■ is cluster 4, and ■ is cluster 5.

Populations labelled ALI, TOM, GGM, DAM, GID, IM2, IM1, GAR, DAY, MUR are from the Arnhem Land Plateau in the Northern Territory and individuals could not be assigned to any one cluster (i.e. they have mixed ancestry from clusters 1, 2 and 4). The population labelled MAC is from the Gulf of Carpentaria and all individuals sampled, except for one, were assigned to cluster 3 with high posterior probabilities. All individuals from EDW assigned with high probability to cluster 5.

Joining tree (Figure 7.4), and clustering of individuals from these sites into one of the two populations were identified by the Geneland analyses (white clusters; Figure 7.5). Overall these results suggest very high levels of gene flow among these upstream populations of the Blyth-Cadel River. The Murrybulljuluk population was significantly different from all other populations based on pairwise θ values but the *Rho* statistics suggested that it was not significantly different from Day (Table 7.2). The Geneland analyses indicated a small probability of genetic exchange between Day and Murrybulljuluk with the boundary of the two populations situated in between these two sites, as indentified by Geneland (Figure 7.5). A second cluster of populations were identified in the Arnhem Land Plateau that comprised the Damdam, Garrom, Thompkinson, and Gidadella sites (Figure 7.5). There was no significant differentiation between Damdam and Garrom ($P > 0.008$), suggesting high levels of gene flow among the downstream regions of the Blyth-Cadel basin. Furthermore, there were no marked genetic differences across adjacent basins as indicated by the lack of differentiation between Garrom and the Thompkinson populations (Table 7.2).The Gidadella population appears to be relatively isolated but with some connections with the Damdam population complex as suggested by its clustering with this group in the Geneland analysis (Figure 7.5) and the genetic similarity to Damdam based on pairwise θ values.

Computer simulations

Computer simulations were used to investigate the effect of an earlier onset of maturity and different harvesting regimes on the genetic diversity of populations in the Blyth-Cadel river basin.

Earlier onset of maturity resulted in an accelerated loss of genetic diversity in the population at Damdam. An effective population size of 500 was required to maintain 90% of the observed alleles when the age of maturity was 6 years, whereas a size of 550 was required to retain the same level of genetic diversity when the age of maturity was 3 years (Table 7.3). Computer simulations indicated that the retention of genetic diversity over the 200 years was directly related to the size of the population. Imimbar retaining the highest levels of genetic diversity (population size = 243; OA = 76.62, *Ho* = 96.69) and Gidadella the lowest levels (population size = 125; OA = 61.78; *Ho* = 93.00) under conditions of no pig predation and no harvesting. All four populations at their current size (i.e. based on 2004 estimates of Fordham et al. 2007a) are expected to lose between approximately 23% to 38% of their allelic richness and a small percentage of heterozygosity (i.e. up to 3%) via genetic drift over the next 200 years even if they are not subject to harvesting or pig predation. A harvest of 20% of the total

Table 7.3 The percentage of the microsatellite genetic diversity retained in the Damdam population over 200 years for various constant population sizes under the scenarios of an age of maturity of three and six years respectively. A_o– number of observed alleles, H_o – observed heterozygosity. Values above 90% are in bold.

	Age of Maturity = 3 years		Age of Maturity = 6 years	
Population Size	A_O	H_O	A_O	H_O
25	40.17	60.63	42.87	63.82
50	46.89	72.35	49.76	75.44
75	52.25	78.99	55.00	81.21
100	56.79	**90.55**	60.09	**92.51**
200	70.69	**95.09**	73.82	**95.95**
300	79.10	**96.76**	82.13	**97.48**
400	84.70	**97.55**	87.22	**98.02**
450	86.91	**97.85**	89.25	**98.25**
500	88.60	**98.21**	**90.80**	**98.46**
550	**90.10**	**98.29**	**92.18**	**98.48**
600	**91.40**	**98.44**	**93.13**	**98.73**

Table 7.4 Percentages of microsatellite genetic diversity retained over 200 years for four populations of Arnhem Land based on computer simulations that account for generation time and overlapping generations.

	Gidadella[1]		Damdam[2]		Murrybulljuluk[3]		Imimbar[4]	
Simulation Scenario	OA	H_O	OA	H_O	OA	H_O	OA	H_O
Not harvested or pig predated	61.78	93.00	64.45	93.65	67.52	95.25	76.62	96.69
20% harvested	56.83	91.61	59.65	92.26	62.60	94.16	71.94	96.04
30% harvested	54.31	90.73	56.72	90.86	59.96	93.15	69.98	95.26
40% harvested	51.23	89.23	53.64	89.47	56.91	92.09	66.34	94.56
Pig predated[†]	48.35	87.74	47.11	85.67	NA*	NA*	NA*	NA*
20% harvested and pig predated	39.41	81.06	41.87	81.50	62.60	94.16	71.94	96.04
30% harvested and pig predated	33.81	74.63	27.00	59.65	59.96	93.15	69.98	95.26
40% harvested and pig predated	25.37	58.19	Extinct[#]	Extinct[#]	56.91	92.09	66.34	94.56

OA – observed number of alleles, Ho – observed heterozygosity.
1 – Gidadella had an initial population size of 125 and an age to maturity of five years based on 2004 estimates of Fordham *et al.* 2007a.
2 – Damdam had an initial population size of 147 and an age to maturity of three years based on 2004 estimates of Fordham *et al.* 2007a.
3 – Murrybulljuluk had an initial population size of 179 and an age to maturity of five years based on 2004 estimates of Fordham *et al.* 2007a.
4 – Imimbar had an initial population size of 243 and an age to maturity of six years based on 2004 estimates of Fordham *et al.* 2007a.
† – Rate of mortality from pig predation was set to 48% for Gidadella, and 58% for Damdam based on estimates of Fordham *et al.* 2006b.
NA* – Not applicable. The presence of pigs is low at Murrybullujulk and Imimbar (Fordham et al. 2006), and thus we have assumed that there is no significant mortality from pigs.
– Simulation can not be performed because the size of the population during the bottleneck was only three individuals and it is presumed to become extinct within 200 years.

population had a small effect on the loss of genetic diversity, with about 5% decrease in allelic richness and 1-2% decline in heterozygosity for all four populations. Harvesting 40% of the total population had a more pronounced effect on loss of genetic diversity, resulting in about 10% loss of allelic richness and up to 5% loss of heterozygosity compared to the levels of diversity retained if the scenario was not-harvested or pig-predated. Pig predation based on mortality rates estimated by Fordham *et al.* (2006) resulted in the greatest loss of genetic diversity compared to all harvesting scenarios with less than 50% of the allelic richness retained in both Damdam and Gidadella. Loss of genetic diversity in pig-predated populations that were subject to harvesting was extreme for all harvesting scenarios. When 40% of the pig-predated population was harvested, only 25% of allelic richness and 58% of heterozygosity was retained for Gidadella. Damdam was predicted to become extinct within 200 years with only three turtles left after accounting for mortality from pig predation and a harvest of 40% of the total population.

Discussion

Genetic diversity estimates were similar for harvested and unharvested populations (Figure 7.1) and no genetic signatures of bottlenecks were detected, even for populations that are frequently harvested. Values differed between the different analyses for pairwise genetic differentiation (Tabe 7.2) owing to differences in their assumptions for mutation. Rho estimates can also perform poorly when low numbers of loci (<20) and sample sizes (<50) and hence the θ analyses (Table 7.2) may be more appropriate for this study (Gagiotii *et al.* 1999).

At a regional scale there were significant and moderate to high levels of genetic differentiation between Arnhem Land populations and those of the Northern Territory (MacArthur River) and Queensland (Edward River) (Table 7.2). In the Arnhem Land region, the East Alligator population was moderately to highly genetically differentiated from all other populations (Table 7.2). Sites downstream of the Blyth-Cadel river basin (i.e. Garrom, Damdam and Gidadella) including the Thompkinson of the Mann/Liverpool river basin were considered to belong to the same cluster (or metapopulation) according to the analysis of Geneland. Downstream sites had non-significant to low levels of genetic differentiation for the pairwise tests between populations (Table 7.2). Sites upstream of the Blyth-Cadel river basin formed a second cluster (or metapopulation) according to the Geneland analysis and had non-significant to low levels of genetic differentiation for the

pairwise tests between populations (Table 7.2). All pairwise comparisons between populations constituting the downstream and upstream metapopulations were significant although the actual values of genetic divergence were relatively low to moderate (Table 7.2) suggesting that the metapopulations are connected via substantial levels of gene flow. Computer simulations lend support to a metapopulation model for *C. rugosa* of the Blyth-Cadel river basin. An effective population size of 500 was forecast to be the size required to maintain 90% of the genetic diversity over 200 years for the Damdam population (Table 7.3); a value that is considerably larger than the 2004 census population size estimate of 147 individuals (Fordham *et al.* 2007). In order for *C. rugosa* populations to have retained the high levels of genetic diversity currently observed they needed to have functioned as a metapopulation with an overall effective population size of at least 500 individuals. Metapopulation genetic structures within river basins have been observed for several freshwater turtle including the diamond terrapins (*Malaclemys terrapin*) of South Carolina (Hauswaldt and Glenn 2005; Tucker *et al.* 2001), neotropical freshwater turtle (*Hydromedusa maximilian)i* of southeastern Brazil (Souza *et al.* 2002a; Souza *et al.* 2002b), ornate box turtles (*Terrapene ornata ornate*) of Iowa (Richtsmeier *et al.* 2008) and the giant Amazon River turtle (*Podocnemis expansa*) (Pearse *et al.* 2006).

Impacts of harvesting

Similar levels of genetic diversity that were found for harvested and unharvested populations suggest that traditional harvesting of *C. rugosa* over millennia has had no adverse impacts on the genetic diversity of populations. Retention of moderate levels of genetic diversity in the populations can be explained by several, possibly interrelated, factors: the intensity and frequency of harvesting; the longevity of the species; compensatory responses to increased mortality; and substantial gene flow among populations. Traditionally, the people were nomadic and would have spread their harvesting efforts thereby avoiding the depletion of populations (Kirk 1981). The long-term settlement of Maningrida was established in 1947 (Maningrida Council 2008) and turtles continue to be harvested in this region (Fordham *et al.* 2008). Sites that are easily accessible to Maningrida and have ideal conditions for harvesting (i.e. are annually dry), such as Damdam, were expected to show signs, if present, of loss of genetic diversity from harvesting. On the contrary, moderate to high levels of genetic diversity were found for easily accessable and frequently harvested populations (Figure 7.1), thus providing strong evidence that harvesting has had no detrimental impacts on the genetic diversity of

populations. The levels of genetic diversity in Arnhem Land populations are also comparable to the unharvested population of Edward River in Queensland (Figure 7.1) suggesting that genetic diversity has not been reduced across all populations of Arnhem Land.

Intensity of *C. rugosa* harvesting may be limited by the traditional methods used to capture them, in which the excavation mounds and breathing holes at the surface of the aestivation chamber are used to locate turtles buried beneath the mud. These signs are not always obvious, particularly if pigs are present and have uprooted the mud under which the turtles aestivate (Fordham *et al.* 2006). Mostly adults and subadults are targeted by traditional harvesting methods while the juveniles, if aestivating, remain undetected (Fordham *et al.* 2006). Harvesting is also regulated by the high inter- and intra-annual variability in the onset and extent of monsoon in tropical Northern Australia (Holland 1986). In years that have higher than average rainfall, the ephemeral waterholes will not dry, turtles will not aestivate and harvest opportunities will be few. Furthermore, the dry season is a period of increased cultural activities that coincide and interfere with turtle harvesting. Fordham *et al.* (2006) estimated that on average a *C. rugosa* population will be subject to traditional harvesting for less than half of all dry seasons (Fordham *et al.* 2006). Intervals of non-harvesting may be an important respite for populations to enable them to partially recover from elevated mortality experienced during harvested years.

Retention of genetic diversity in harvested populations of *C. rugosa* may also be due to its longevity; a trait which buffers the loss of genetic diversity via genetic drift (discussed in detail in Chapter 2). Long-lived species typically lose less genetic diversity during a population bottleneck than short-lived species because for the duration of time that the bottleneck persists they have fewer generations (Diaz *et al.* 2000; Kuo and Janzen 2004). Traditional harvesting of populations has been conducted for millennia, however if it has been conducted at low intensity it is expected that little genetic diversity will be lost. Diffuse bottlenecks (i.e. from low intensity harvesting) that occur over several generations results in less loss of genetic diversity compared to short intense bottlenecks (i.e. from intense harvesting) (England *et al.* 2003). The genetic results for this study are consistent with genetic studies of other long-lived species that have retained high levels of genetic diversity despite long and persistent population bottlenecks, such as the ornate box turtle

(Kuo and Janzen 2004), giant Amazon River turtle (Pearse *et al.* 2006), black rhinoceros (Swart *et al.* 1994), and white-tailed eagle (Hailer *et al.* 2006).

Compensatory responses to elevated mortality such as an earlier onset of maturity have been considered to alleviate the impacts of harvesting pressures (Abrams and Rowe 1996; Poysa *et al.* 2004). Fordham *et al.* (2007) demonstrated a compensatory response in the frequently harvested population of Damdam with an earlier onset of maturity in females of three years of age compared to five to six years in unharvested populations. Whilst an earlier onset of maturity reduces extinctions risks from demographic stochasticity by stabilising the size of the population it has a negative effect on genetic diversity. Simulations forecast that an effective population size of at least an extra 50 individuals are required to maintain 90% of the total genetic diversity over 200 years when the age of maturity is three years compared to six years of age (Table 7.3). Reducing the age of maturity shortens the generation time and thereby makes it more vulnerable to loss of genetic diversity if a great number of generations pass through population bottlenecks. Hence, the compensatory response of an earlier onset of maturity can not explain the retention of high levels of genetic diversity in the study populations that were subject to harvesting. Mutation may also contribute to genetic diversity within populations. Further, other compensatory responses such as increased hatchling recruitment have been demonstrated for the study populations (Fordham, unpublished data) and may have a positive effect on the retention of genetic diversity for harvested populations.

Local distribution patterns of *C. rugosa* in the Blyth-Cadel river basin and its dispersal capability will greatly influence the levels of genetic diversity for harvested populations. *Chelodina rugosa* is widespread in the Blyth-Cadel river basin and has been documented from over 80 sites (Fordham, unpublished data). During the wet season that extends from December to March, extensive wetlands form over an area of approximately 100, 000 hectares across the Blyth-Cadel and Mann-Liverpool River systems (Finlayson *et al.* 1999). These wetlands provide plenty of opportunity for dispersal of turtles across sites within the Blyth-Cadel basin and even across river basins. In addition, compared to other Australian freshwater turtles, *C. rugosa* is highly adept at dispersing across land and has been known to travel over two kilometres before reaching a water-body (Fordham, unpublished data*). Cheldoina rugosa* in the Blyth-Cadel river basin exhibited a

metapopulation structure characterised by low levels of genetic differentiation between populations (Table 7.2).

A metapopulation structure can reduce the impacts of harvesting on any one population by increasing the effective population size (i.e. the number of reproductively contributing individuals) and hence more individuals can be harvested before there are any detrimental effects (Allendorf *et al.* 2008; Bischof *et al.* 2008; Coltman 2008; McCullough 1996; Tenhumberg *et al.* 2004). Connectivity of non-harvested and harvested sites via gene flow would provide 'genetic rescue' for populations, whereby novel genetic material is introduced into populations by immigrants from other populations (Ingvarsson 2001; Tallmon *et al.* 2004). Even low levels of gene flow of one to ten immigrants per generation is sufficient to ameliorate the effects of inbreeding in a bottlenecked population and thereby increase the fitness of the population (Ingvarsson 2001; Tallmon *et al.* 2004: also see Chapter 2). In the absence of gene flow among populations, the simulations forecast that a harvesting regime of 20% of the total population had minimal impact on genetic diversity of populations but the impact was considerable when 30 to 40% of the population was harvested (Table 7.4). If gene flow among populations occurs on a regular basis, then harvest regimes that remove more than 20% of the population would still be sustainable and hence the overall functioning of the metapopulation, would not be compromised. Fordham *et al.* (2008) recommended that a 30% harvest of the sub-adult and adult population that occurs in half of all dry years is sustainable based on demographic models. Effects of harvesting can be exacerbated by elevated natural mortality, such as from prolonged drier years or pig predation, that can result in the overexploitation of the population even when harvest intensities are low. Simulations indicated that even low intensity harvesting of populations that are subject to pig predation resulted in substantial loss of genetic diversity (Table 7.4). Gene flow among populations would reduce the loss of genetic diversity by providing genetic rescue to populations that are subject to pig predation.

Implications for conservation

Based on the genetic data and computer simulations it appears that traditional harvesting has had minimal impact on the genetic diversity of *C. rugosa* populations of the Blyth-Cadel region. The harvesting regimes and longevity of the species may have aided in the retention of genetic diversity of harvested populations. Furthermore, it appears that high

levels of connectivity and gene flow among populations have provided genetic rescue to harvested populations and retarded the loss of genetic diversity. Maintaining high levels of gene flow and functioning of the metapopulation is important to maintain genetic diversity, and hence adaptive potential for *C. rugosa* in the Blyth-Cadel region. Harvesting can be conducted sustainably provided that gene flow between populations is maintained. If gene flow becomes disrupted and results in the collapse of the metapopulation simulations forecast substantial loss of genetic diversity for all populations over 200 years (approximately 30% - 40% of allelic richness and 5-10% of expected heterozygosity; Table 7.4) even for those that are not subject to harvesting or pig predation. Ongoing genetic monitoring of the harvested and unharvested populations can be used to measure the contemporary levels of gene flow across the landscape to ensure that harvesting is not having an adverse effect on the overall functioning of the metapopulation.

Demographic-based model projections of Fordham (2008) indicate that *C. rugosa* population are under severe threat from pig predation and are likely to become extinct within the next 30 years unless actively managed. This situation is dire; worse than the effect of pig predation forecast from the genetic data that was simulated with the worst-case scenario of a metapopulation collapse and subsequent isolation of populations (Table 7.4). Thus populations subject to pig predation are at greater risk of extinction from demographic rather than genetic processes. Hence, as a priority, populations need to be managed to reduce the effects of pig predation either via localised culling of pigs, fencing off waterholes, or headstarting turtles to mitigate the elevated adult mortality (Fordham *et al.* 2008). For areas that have been managed to mitigate the impacts of pig predation, sustainable harvesting can be achieved provided that gene flow is maintained, either through natural or artificial means, between harvested and unharvested populations of the Blyth-Cadel basin. Depleted populations can be restocked with turtles sourced from a broad geographic range in the Blyth-Cadel basin.

Chapter 8 – DNA-based identification of wildlife to species, region and population of origin: A case study using the Australasian snake-necked turtle *Chelodina rugosa*.

Picture: The study species – *Chelodina rugosa* – for which a DNA-based forensic identification system was developed. Photo by Erika Alacs.

Chapter 8 – DNA-based identification of wildlife to species, region and population of origin: A case study using the Australasian snake-necked turtle *Chelodina rugosa*.

Abstract

An hierarchical approach was used to develop a DNA-based system for the forensic identification of wildlife seizures to species, region and population specific origin using the Australasian snake-necked turtle *Chelodina rugosa* as a case study. An 898 bp region of the nuclear R35 intron discriminated *Chelodina rugosa* from all other Australian chelid turtles. Hybrids can be a challenge for the accurate species identification of wildlife seizures. A combination of sequence for 867bp of the mitochondrial DNA (control region and ND4) and 17 microsatellite loci demonstrated natural hybridization between *C. burrungandjii* and *C.rugosa*, and between *C. canni* and *C. rugosa*. An F2 *C. burrungandjii* and *C.rugosa* hybrid individual, and two *C. burrungandjii* backcrossed individuals (i.e. hybrid x *C. burrungandjii)* were identified by the microsatellite analysis. Geographic sources of specimens could be reliably assigned to three distinct regions using sequencing of 867 bp of the mitochondrial DNA: (i) Darwin (Finnis basin), (ii) Arnhem Land, and (iii) eastern Queensland including southern New Guinea. Specimens could not be identified to a source locality at the population-level in the Blyth-Cadel basin of Arnhem Land where a commercial trade has been established. Poor population assignment of individuals to specific localities in the basin based on genotypes for 12 unlinked microsatellite loci is most likely caused by high levels of gene flow between sites in the Blyth-Cadel basin. A DNA-based forensic identification system to identify seizures of *Chelodina rugosa* to the species level and to determine provenance at the broad landscape scale is feasible, but assignment within more restricted regions linked by coastal floodplains is probably not. Given the isolation and inaccessibility of the Arnhem Land region, this level of identification may be adequate to verify the legality of specimens from the commercial industry.

Introduction

The illegal trade in wildlife has been reported by International Policing (Interpol) to be a serious and growing global crime worth more than USD $20 billion per year (Interpol, 2007). While DNA technologies have been routinely used to identify humans and provide

evidence for crime since 1985 (Gill *et al.* 1985; Jeffreys *et al.* 1985a; 1985b; Reeder 1999), their application to provide evidence for wildlife crime is still in its infancy. DNA can be extracted from degraded or highly processed products that are commonly encountered in illegal wildlife trade, such as from cooked meat (Martinez and Danielsdottir 2000), powdered bone (Prado *et al.* 2002), claws left on tanned hides (Hedmark and Ellegren 2005), egg shells (Moore *et al.* 2003), hair (Jedrzejewksi *et al.* 2005; Savolainen and Lundeberg 1999) and feathers (Rudnick *et al.* 2007). Information can be obtained on what species or composite of species the seizure has been derived from (Chapman *et al.* 2003; Hsieh *et al.* 2003; Huang *et al.* 2003; Ludwig 2008). Geographic origins of the seizure can be determined and used to distinguish between legal and poached specimens (Stam *et al.* 2006; Waldman *et al.* 2008; Withler *et al.* 2004), and to identify 'hotspots' of poaching activities where wildlife enforcement and policing need to be strengthened (Wasser *et al.* 2008; Wasser *et al.* 2007). DNA technologies have been used successfully, to date, to provide evidence for cases of wildlife poaching by identification of seizures (or evidence such as blood on a knife) to the species level (Lorenzini 2005; Pitra and Lieckfeldt 1999), their geographic origins (Withler *et al.* 2004), sex (An *et al.* 2007) and parentage (An Lee *et al.* 2007).

DNA technologies for wildlife forensics have mostly been developed on a case-by-case basis. The development of DNA technologies to provide evidence for individual wildlife cases is often expensive and time-consuming, particularly when there is little or no prior genetic information on the species of interest. This is more often than not the situation faced with when dealing with wildlife cases. Challenges also arise in the development of forensic identification systems for wildlife. Species identification of wildlife can be difficult when species boundaries are poorly known, cryptic species that are morphologically indistinguishable but genetically different are present, or when inter-specific hybridisation and introgression occurs, in which the fertile hybrids mate with parental species thus resulting in complex patterns of genetic inheritance (Adams and Waits 2007; Allendorf *et al.* 2001; Arnold 1992; Belfiore *et al.* 2003; Goldstein *et al.* 2005; Sites and Marshall 2003). These issues have often been overlooked in the development of DNA-based systems to identify wildlife species but can be overcome with appropriate choice of markers and approach.

Identification of the geographic origins of seizures to either the regional or population levels first requires extensive sampling across the landscape to detect genetic structure. The geographic scale at which genetic structure becomes apparent will depend on the spatial distribution of the populations and the propensity of individuals to disperse across the landscape, and hence will determine the resolution (in terms of distance) that the source locality of the specimen can be identified to (Cornuet *et al.* 1999a; Manel *et al.* 2005). Taking into consideration these issues, this study aims to develop a DNA-based system for identification of wildlife seizures to species, regional and population levels for purposes of monitoring trade and provision of evidence for relevant wildlife cases.

A hierarchical approach was adopted whereby a seizure is first identified to the species level, and if it is identified as being derived from the species of interest it is subject to further DNA analysis to identify its regional and population origins. This approach can be applied to any species of interest and has the advantage that it employs several different DNA methodologies for identification that when used in concert provides strong and convincing evidence for wildlife cases. The methods employed for each level of identification were chosen based on their general acceptance in the scientific community, presence of well established protocols and analysis techniques established for other disciplines such as molecular systematics and conservation genetics, and their past success in being accepted as evidence for court cases (An *et al.* 2007; Lorenzini 2005; Pitra and Lieckfeldt 1999; Withler *et al.* 2004).

The species used for the case study is the Australasian snake-necked turtle *Chelodina rugosa*; a common and locally abundant freshwater turtle inhabiting rivers and ephemeral waterholes of northern Australia and southern New Guinea (Georges *et al.* 2002; Manning and Kofron 1996). Commercial trade of *Chelodina rugosa* to supply hatchlings to the pet shop industry was established in 2003 by the Bawinanga Aboriginal Community in the town of Maningrida situated on the Blyth-Cadel basin of Arnhem Land. Identification of the geographic origins of seizures of *Chelodina rugosa* is important for distinguishing between animals derived from the legal trade in Maningrida and those that have been poached from other locations within the species range. Many of the challenges and issues for the development of DNA-based forensic identification systems are embodied in the chosen species, *C. rugosa*: it is known to hybridise with two sympatrically distributed species *Chelodina burrungandjii* and *Chelodina canni* (Georges *et al.* 2002), it has a

widespread distribution across Northern Australia and New Guinea where it is locally abundant (Cann 1998), and it has good dispersal capability through water channels and across land (Fordham *et al.* 2007). It is therefore expected that many of the issues that can arise for DNA-based forensic identification will be highlighted in this study and will be of use to inform future developments of DNA-based forensic identification systems for wildlife.

Methods

Species identification using the nuclear R35 intron phylogeny.

Species from the family Chelidae distributed in the Australasian region were sequenced with 898-bp of the nuclear R35 intron. One sample from each clade of species with control region or ND4 region sequence (Chapter 7; Georges unpublished data) was included to capture the genetic variability within species. The data set consisted of 45 individuals representing 25 species from five genera in Australasia (Figure 8.1). Three taxa were used as outgroups for the phylogrny: two chelids; *Phyrnops nasuta* from Surinam and *Hydromedusa tectifura* from Uraguay, and one-non chelid; *Erymnochelys madagascariensis* from Madagascar (Figure 8.1).

We performed DNA extractions using a standard Chelex protocol for skin samples (Walsh *et al.* 1991) or salt extraction protocol for blood samples (Sambrook and Russell 2001). Sixteen samples representing two species from each of the eight genera were sequenced with the primers R35Ex1 and R35Ex2 according to the PCR protocols described in Fujita *et al.* (2004). The sixteen sequences were aligned and used to develop an internal primer called R35int. Approximately 503-bp of the R35 region was amplified using the primers R35Ex1 (5′ ACG ATT CTC GCT GAT TCT TGC) (Fujita *et al.* 2004) and R35intR (5′ ATG GAA AGR AGC TGA NAG G). A second fragment of approximately 395 bp of the R35 region was amplified using the primers R35intF (5′ CCT NTC AGC TYC TTT CCA T) and R35Ex2 (5′ GCA GAA AAC TGA ATG TCT CAA GG) (Fujita *et al.* 2004). Both reactions contained 50-100 ng of template DNA, 0.25 μM of each primer, 0.0625 μM each dNTP, 1.5 mM $MgCl_2$, 2.5 μl 10 × PCR buffer, 0.6 M betaine, 5 μg BSA and 0.51 U *Taq* DNA polymerase (Bioline Red *Taq*) in a total volume of 25 μl.

Table 8.1 Samples of Australasian chelid turtles used in the nuclear R35 phylogeny.

Species	**Specimen**	**State and Country**	*River Locality*
Elusor macrurus	281	NSW, Australia	Mary
Elseya latisternum	AA1579	QLD, Australia	Burnett
Elseya latisternum	AA20125	NT, Australia	Limmen Bight
Elseya georgesi	EG154	NSW, Australia	Bellinger
Elseya purvisii	EP130	NSW,	Manning
Elseya purvisii	EP129	NSW,	Manning
Elseya novaeguineae	AA42030	West Papua	Aru Island
Elseya novaeguineae	AA42029	West Papua	Waren
Elseya albagula	AA271	QLD, Australia	Burnett
Elseya dentate	212	NT, Australia	South Alligator
Elseya dentate	713	NT, Australia	Limmen Bight
Elseya dentate	AA20063	NT, Australia	Roper
Elseya branderhorsti	AA42608	Papua New Guinea	Fly
Elseya albagula	AA17780	QLD, Australia	Mary
Elseya irwini	EI01	QLD, Australia	Burnett
Elseya irwini	EI02	QLD, Australia	Burnett
Rheodytes leukops	232	QLD, Australia	Fitzroy
Emydura macquarii krefftii	AA20882	QLD, Australia	Burdekin
Emydura macquarii macquarii	132	NSW, Australia	Clarence
Emydura macquarii macquarii	AA13051	QLD, Australia	Warrego
Emydura macquarii nigra	50L	QLD, Australia	Fraser Island
Emydura tanybaraga	AA13610	QLD, Australia	Stratton
Emydura tanybaraga	AA13632	QLD, Australia	Mitchell
Emydura victoriae	254	WA, Australia	Victoria
Emydura worrelli	AA20069	NT, Australia	Roper
Emydura worrelli	AA20138	NT, Australia	MacArthur
Emydura subglobosa	AA42623	Papua New Guinea	Fly
Chelodina longicollis	CL72	NSW, Australia	Mary
Chelodina longicollis	AA20521	QLD, Australia	Burnett
Chelodina canni	AA20248	QLD, Australia	Mitchell
Chelodina pritchardi	497	Papua New Guinea	Kemp
Chelodina novaeguineae	456	Papua New Guinea	Aramia
Chelodina reimanni	491	Papua New Guinea	Merauke
Chelodina oblonga	398	WA, Australia	Swan
Chelodina burrungandjii	AL018	NT, Australia	Roper
Chelodina burrungandjii	AL004	NT, Australia	Cadel
Chelodina spp. (Kimberley)	N1721	WA, Australia	Durack
Chelodina spp. (Kimberley)	N1208	WA, Australia	Drysdale
Chelodina spp. (Kimberley)	N624	WA, Australia	Isdell
Chelodina rugosa	G85	QLD, Australia	Holroyd
Chelodina rugosa	478	West Papua	Binituri
Chelodina rugosa	AA20078	NT, Australia	Roper
Chelodina rugosa	AA20002	NT, Australia	East Alligator
Chelodina rugosa	Y007	NT, Australia	Robinson
Chelodina expansa	CE107	NSW, Australia	Murray
Phyrnops nasuta	838	Surinam	Unknown
Erymnochelys madagascariensis	850	Madagascar	Unknown
Hydromedusa tectifera	587	Uraguay	Rio Santa Lucia

cycling conditions used to amplify both sections of the R35 intron were an initial denaturation of 94 ^{o}C for 5 min, followed by 35 cycles of 94 ^{o}C for 30s, 58 ^{o}C for 90s, 72 ^{o}C for 120s, and a final extension of 72 ^{o}C for 10 min. Polymerase chain reaction products were purified using a standard polyethylene glycol (PEG) procedure (Sambrook and Russell 2001) and sequenced using an ABI automated sequencer at the Macrogen facility in Seoul, Korea.

Sequence data were edited using SEQUENCHER 4.2.2 (Gene Codes Corporation) and then aligned using CLUSTAL X (Thompson *et al.* 1997) in the program Geneious Pro 3.8.2 (Biomatters Ltd). Alignments were verified by eye. The primer ends were removed from the two alignments of the 503 bp and 395 bp regions of the R35 intron and then the alignments were concatenated for further analysis. Indels were binary coded and unweighted. Maximum parsimony (MP) and maximum likelihood analyses were conducted on the concatenated alignment in the program PAUP 4.0b10 (Swofford 2000). The MP analysis assumed that character changes were unordered and of equal weight and used a random stepwise sequence addition algorithm with tree-bisection-reconnection (TBR) branch swapping. Modeltest 3.7 (Posada and Crandall 1998) was used to determine the best fit model of sequence evolution. Modeltest 3.7 (Posada and Crandall 1998) determined that the best fit model of sequence evolution based on Akaike Information Criterion (AIC_c) was the TVM with gamma distribution (G) of rate heterogeneity across variable sites. The estimated parameters under this model with a gamma shape distribution of 0.6018 were implemented in the ML analysis conducted in PAUP 4.0b10 (Swofford 2000). Bootstrap replicates of 1000 for both MP and ML were conducted to estimate nodal support. The trees were rooted with *Phyrnops nasuta*, *Hydromedusa tectifera*, and *Erymnochelys madagascariensis*. Sequence divergences (*p*) values were estimated between species, and were corrected for within phylogroup diversity (Avise and Walker 1998).

Regional identification using mitochondrial phylogeography of C. rugosa.

The sequencing and phylogenetic analysis for 867 bp of the mitochondrial control and ND4 regions for specimens of *Chelodina rugosa* across its range (Figure 8.1) have been described in detail in the methods section of Chapter 6.

Identification of hybrids using microsatellites.

A total of five *C. burrungandjii* and 55 *C. rugosa* individuals were genotyped for 17 microsatellite loci. *C. burrungandjii* samples represented three major drainages: South Alligator River (one sample), Blyth-Cadel River (three samples), and Roper River (one samples). Samples of *C. rugosa* were from the Blyth-Cadel River (46 samples from 10 localities), Roper River (two samples) and East Alligator River (seven samples). Microsatellite genotyping and analysis has been described in detail in Chapter 6.

Population identification using microsatellite markers.

A total of 311 tissue samples of *Chelodina rugosa* were collected from 2000 to 2005 from eight sites in the Blyth-Cadel basin of Arnhem Land, Northern Territory, Australia (sample size, population abbreviation, and GPS location in parenthesis): Gidadella (GID; *N* = 33; 12° 31' 33" S, 134° 21' E), Imimbar 1 (IM1; *N* = 41; 12° 44' S, 134° 31' 48" E), Imimbar 2 (IM2; *N* = 35; 12° 44' 37" S, 134° 32' E), Garromgarrom (GGM; *N* = 28; 12° 18' 54" S, 134° 30' 23" E), Garoada (GAR; *N* = 14; 12° 40' 11" S, 134° 41' 43" E), Damdam (DAM; *N* = 34; 12° 9' 53" S, 134° 37' 36" E), Day (DAY; *N* = 18; 12° 37' 08" S, 134° 43' 02" E), and Murrybulljuluk (MUR; *N* = 36; 12° 36' 59" S, 134° 51' 42" E). In addition, samples were collected in 2004 from two basins adjacent to the Blyth-Cadel basin: Thompkinson River (TOM; *N* = 32; 12° 12' 49" S, 134° 17' 01" E), and East Alligator River (ALI; *N* = 9; 12° 14' 55" S, 133° 8' 50" E). Northern Queensland samples were represented from two localities collected in 2004: MacArthur River (ART; *N* = 18; 15° 56' 20" S, 136° 12' 30" E), and Edward River (EDW; *N* =13; 14° 50' S, 142° 9' 20" E) (Figure 8.2).

DNA was extracted from small slithers of skin from the clawless hind toe using a standard salting–out protocol (Dethmers *et al.* 2006). Samples were genotyped for unlinked loci as determined in Chapter 6: four dinucleotide loci (T-11, T-31, T-41, T-47) and eight trinucleotide loci (T-12, T-14, T-17, T-42, T-44, T-58, T-80, T-87). The isolation of the microsatellites, primer design, conditions for multiplex polymerase chain reaction (PCR), and separation of fragments on the Beckman Coulter CEQ 8000 Genetic Analysis System have been described previously in Chapter 5.

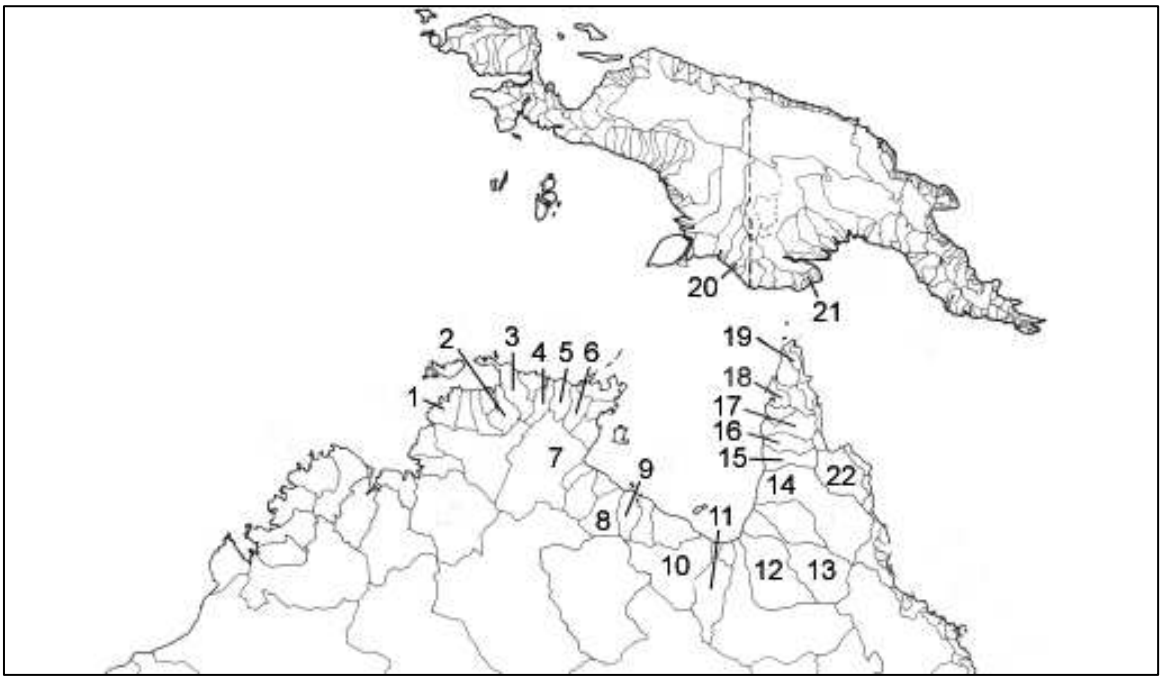

Figure 8.1. Australian, New Guinea and West Papua drainage basins showing the 22 basins from which *Chelodina rugosa* samples were collected for the mitochondrial phylogeographic study. Major drainage basins are numbered as follows: **1.** Finnis, **2.** South Alligator, **3.** East Alligator, **4.** Liverpool, **5.** Blyth/Cadel, **6.** Goyder, **7.** Roper, **8.** MacArthur, **9.** Robinson, **10**. Nicholson, **11.** Leichardt, **12.** Norman, **13.** Gilbert, **14.** Mitchell, **15.** Edward, **16.** Holroyd, **17.** Archer, **18.** Wenlock, **19.** Jardine, **20.** Merauke, **21.** Binituri, **22.** Normanby. (Figure taken from Chapter 6).

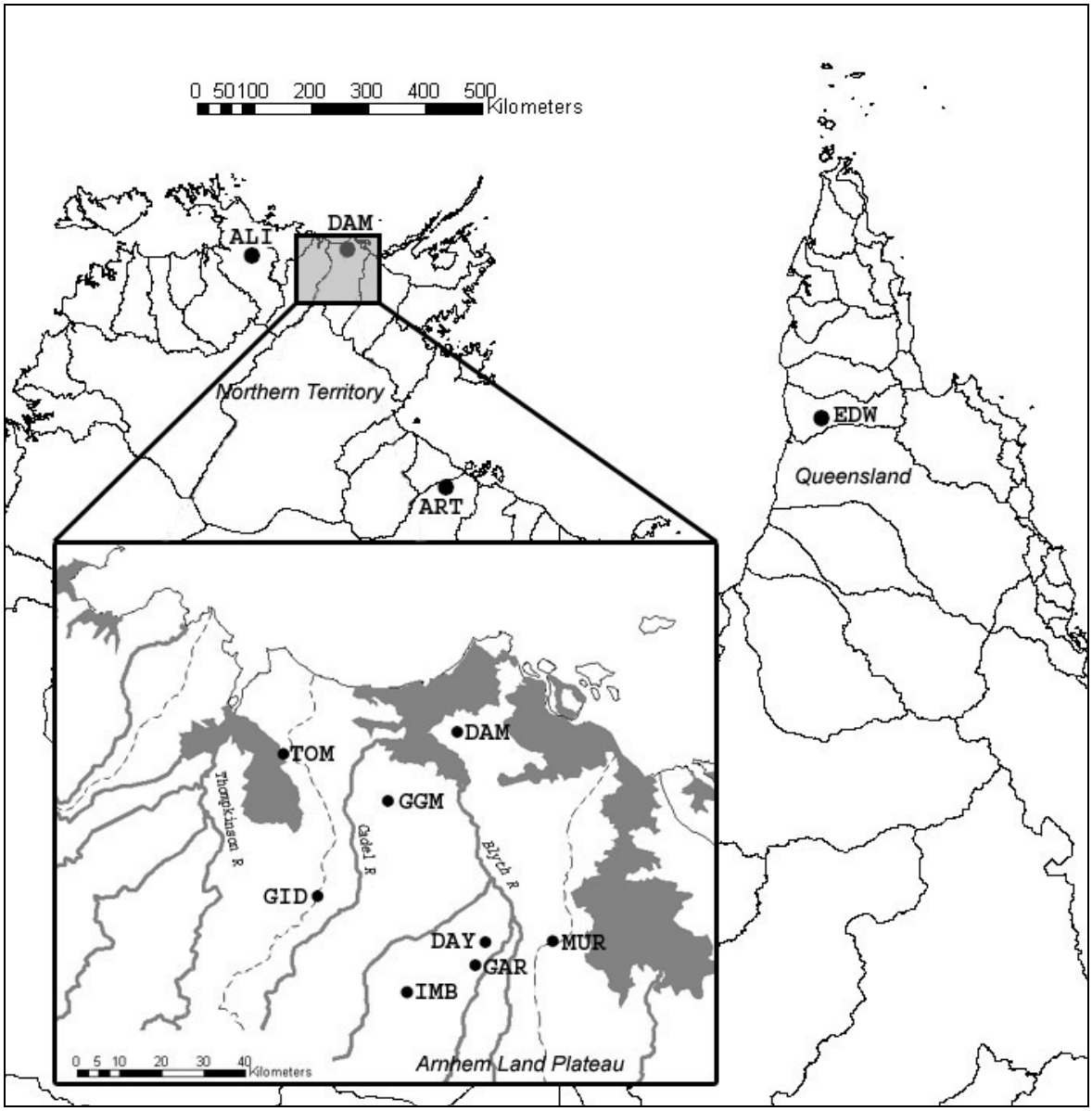

Figure 8.2 Map of the major river basins of northern Australia indicating sampling locations for population assignment analyses of *C. rugosa*. Insert shows sampling in the Blyth-Cadel and Mann-Liverpool basin (basin boundaries indicated by dashed lines) of northeast Arnhem Land, Northern Territory. Shaded regions indicate ephemeral floodplains. (Figure taken from Chapter 7).

The presence of genotyping errors, such as scoring of stutter peaks, non-amplification of null alleles and dominance of smaller alleles were assessed with MICRO-CHECKER software (Van Oosterhout *et al.* 2004). Assignment of individuals to populations was assessed using four different methods: (i) frequency-based assignment using simulations to estimate the probability of individuals belonging to predefined populations in the program GeneClass2 (Piry *et al.* 2004), (ii) bayesian-based assignment using simulations to estimate the probability of individuals belonging to predefined populations in the program GeneClass2 (Piry *et al.* 2004), and (iii) bayesian clustering algorithms to firstly identify how many populations (i.e. clusters) are present based on genotypic frequencies and then the probability of individuals belonging to each of these clusters using the program Structure v2.2 (Pritchard *et al.* 2000).

For Geneclass2, both the frequency-based (Paetkau *et al.* 1995) and bayesian-based assignment methods (Rannala and Mountain 1997) used the resampling algorithm of Cornuet *et al.* (1999b) to estimate the probabilities of individuals belonging to a predefined population with simulations of 1000 individuals and a maximum type I error of 0.01. To asesss the probability of individuals being correctly assigned their river basin of origin the three predefined populations (or priors) chosen were: the Blyth-Cadel River including the Thompkinson (Arnhem Land, NT; *N = 271*), MacArthur River (Gulf of Carpentaria, Qld; *N = 18*), and Edward River (Cape York, Qld; *= 13*). The Thompkinson samples were grouped with those of the Blyth-Cadel based on the high levels of gene flow found between these adjacent basins for 12 microsatellite loci (see Chapter 7). The probability of individuals being correctly assigned to a specific site within the Blyth-Cadel basin (including the Thompkinson) was assessed using the following predefined populations (or priors): eight populations within the Blyth-Cadel basin including one population from the adjacent Thompkinson basin; denoted as TOM (*N = 32*), GID (*N = 33*), IM1 (*N = 41*), IM2 (*N = 35*), GGM (*N = 28*), GAR (*N = 14*), DAM (*N = 34*), DAY (*N = 18*) and MUR (*N = 36*) (Figure 8.2).

For the Structure analysis, bayesian clustering methods were used employing an admixture model, in which individuals can have mixed ancestry with genotypic contributions from different populations (Pritchard *et al.* 2000). The admixture model was run for K = 1 to K = 12 clusters. Each run using the Markov Chain Monte Carlo (MCMC) sampling was for 1 million iterations with a burning in period of 100,000 iterations. The K that bests

represented the data was chosen by plotting log Pr(X | K) for five runs of each K from 1 to 12 and then choosing the K at which the plot reaches an asymptote as recommended by (Pritchard *et al.* 2000). The analysis was then run ten times at K = 5 which was the number of clusters that was found to best represent the data. Q-values which are the posterior probabilities of individuals belonging to each of K clusters were plotted.

Results

Species identification using the nuclear R35 intron

The final alignment consisted of 898 bp with 379 variable sites, of which 126 were phylogenetically informative. Indels ranged in size from one to 30 bp. The MP and ML analyses produced gene trees with similar topologies and high bootstrap support (Figure 8.3). There were 24 unique haplotypes, two of which were shared across different species. One haplotype was shared for the closely related species *Emydura worrelli*, *E.victoriae* and *E. subglobosa*. The second haplotype was shared for closely related species *Chelodina pritchardi*, *C. reimanni* and *C. novaeguineae*. The genus *Elseya* had two clades; one comprised of *Elseya belli*, *E. latisternum* (Queensland), *E. georgesi* and *E. purvisi* that was 1.2% divergent from the second clade comprised of *Elseya latisternum* (Northern Territory), *Elseya dentata*, *Elseya branderhorsti*, *Elseya albagula* and *Elseya irwini*. *Rheodytes leukops* was closely associated with the second clade. These two clades were paraphyletic, in that the first clade (comprised of *Elseya belli*, *E. latisternum* (Queensland), *E. georgesi* and *E. purvisi*) was more closely related to the *Emydura* clade than to the second clade (comprised of *Elseya latisternum* (Northern Territory), *Elseya dentata*, *Elseya branderhorsti*, *Elseya albagula* and *Elseya irwini*). *Elseya latisternum* was unusual in that it had two very divergent haplotypes – one from Queensland and other from the Northern Territory – that grouped into different *Elseya* clades, suggesting that they may represent two distinct taxa.

There was strong support for the *Chelodina* as a clade (bootstrap support 100 for both MP and ML analysis) approximately 2% divergent from the clade of *Emydura* and two clades of *Elseya*. There were two clades within the *Chelodina* clade – *Chelodina pritchardi*, *C. reimanni*, *C. novaeguineae*, *C. longicollis* and *C. canni* formed one clade. *Chelodina burrungandjii*, *C. rugosa* and *C. expansa* formed the second clade.

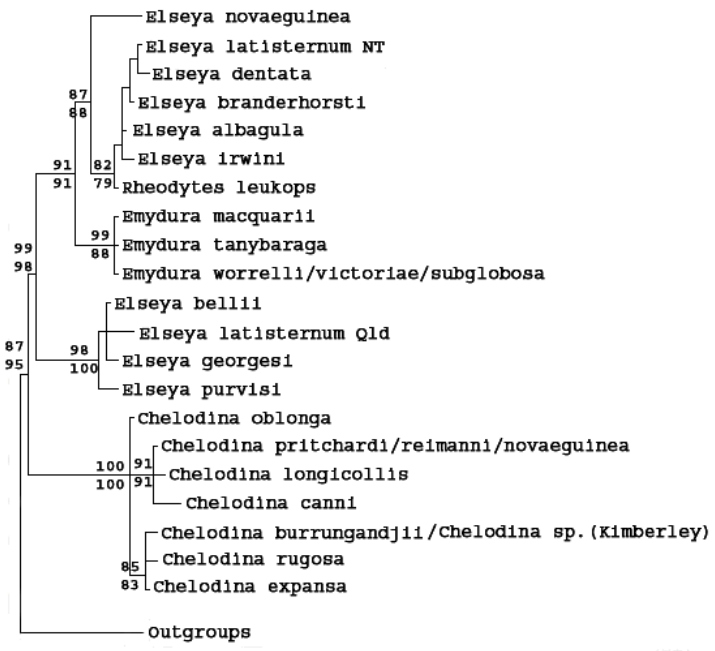

Figure 8.3. Phylogenetic analysis of Australasian chelid turtles for 898-bp of the nuclear R35 intron. Node support shown for maximum parsimony is based on 1000 bootstrap replicates and maximum likelihood with 1000 bootstrap replicates.

Chelodina oblonga could not be placed in either clade and its relationship could not be resolved. All samples of *Chelodina rugosa*, including those from New Guinea (formerly *Chelodina seibenrocki*), shared the same haplotype. Similarly *C. burrungandjii* from Arnhem Land and Chelodina sp. (Kimberley) shared the same haplotype and differed from *C. rugosa* by one fixed site (A↔G transversion).

Regional identification using the mitochondrial phylogeography of C.rugosa.

The final alignment of 867 bp had 224 variable sites and 204 parsimony-informative sites. Seven indels were present within the mitochondrial control region. Gene tree topologies for MP and ML analyses were similar. There were 41 haplotypes for *C. rugosa* that fell into two well supported major lineages that were 2.4% divergent from each other (Figure 8.4). One lineage comprised of haplotypes from the Finnis River to the Roper River of the Northern Territory, including those of the Blyth-Cadel basin (West Lineage; Figure 8.5). Within this West Lineage, specimens from the Finnis formed a clade that was sister to a clade comprising specimens from Arnhem Land (East Alligator, South Alligator, Blyth-Cadel, Goyder and Roper Rivers) with strong bootstrap support of 99% and 95% for MP and ML analysis respectively (Figure 8.4). The second lineage (named the East Lineage) comprised of samples from the Gulf of Carpentaria, Cape York and southern New Guinea (Figure 8.5). There was no significant phylogeographic structuring within the East Lineage, with some haplotypes shared across river basins (Figure 8.5).

Identification of hybrids

The existence of natural hybridization presents obvious complications for use of DNA approaches to species assignment in wildlife forensics. The mitochondrial gene tree found evidence of hybrids. Haplotypes that had *C. rugosa* and *C. burrungandjii* morphologies formed a clade that was sister to and 4.85% divergent from the *C. rugosa* West Lineage (Figure 8.4). The *C. burrungandjii* mitochondrial haplotype was found in the South Alligator, Cadel, and Roper Rivers. Microsatellites independently confirmed the hybrid origins of this clade, by identifying one of the haplotypes as having a high posterior probability of being an F2 hybrid (84%) and two others of having weak signatures (24% and 22% probabilities respectively) of being a backcross produced by a fertile *C. burrungandjii* x *C. rugosa* hybrid mating with a male *C. burrungandjii*.

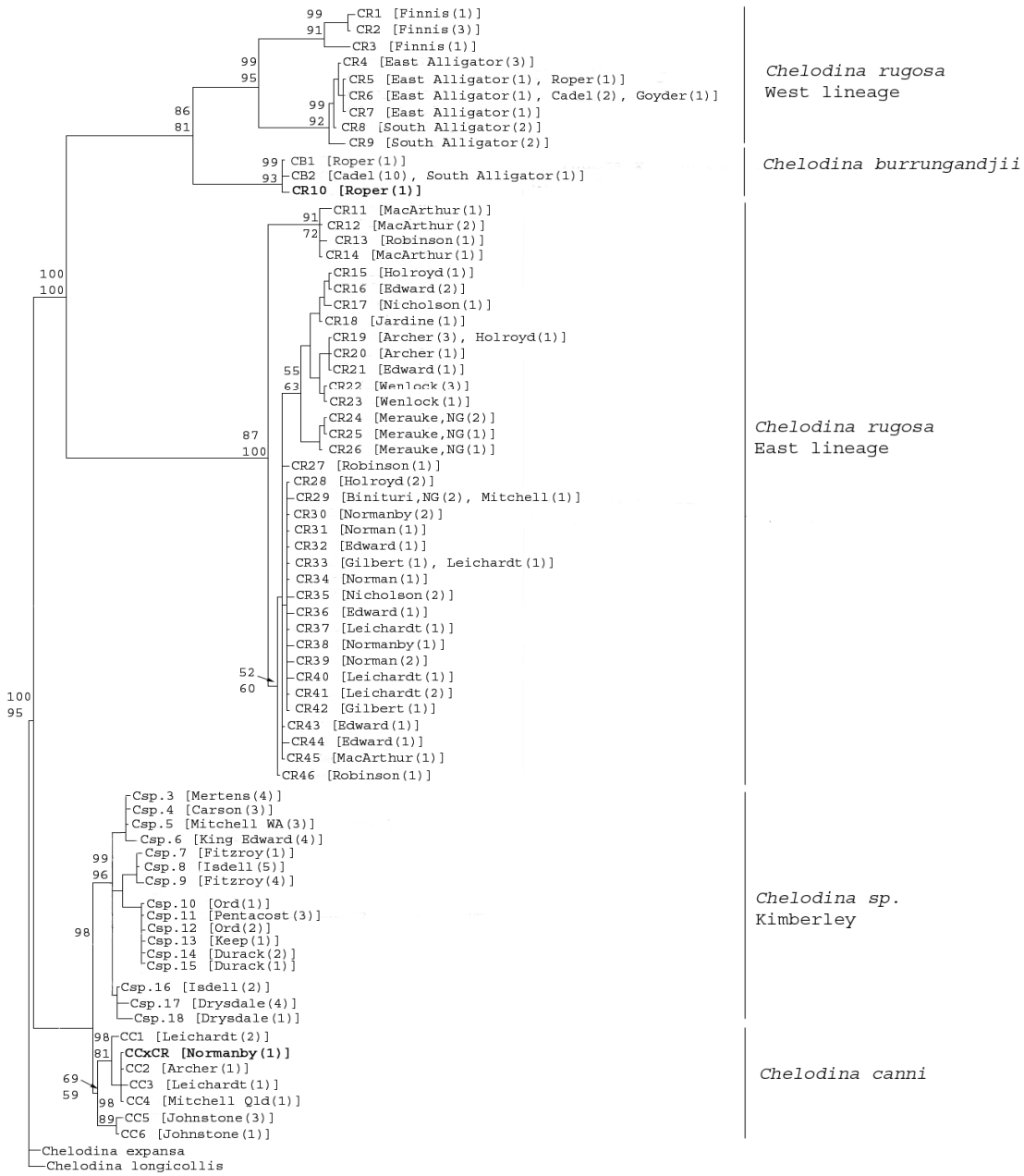

Figure 8.4 Phylogenetic analysis of *Chelodina rugosa*, *C. burrungandjii*, *Chelodina sp.* (Kimberley) and *C. canni* based on 867 bp from the mitochondrial ND4 and control region. Haplotypes are labelled as: the species based on morphology (i.e. CR = *Chelodina rugosa*, CB = *C. burrungandjii*, Csp. = *Chelodina sp.* (Kimberley) CC = *C. canni*\. Following the haplotype name is information of their localities to main river basins and the sample size in parentheses for each locality. Numbers at nodes refer to bootstrap values with 1000 and 100 replicates performed for Maximum Parsimony (value above) and Maximum Likelihood (value below) analyses respectively. Haplotypes in bold have a morphology that is characteristic for a different species compared to their haplotype. (Figure taken from Chapter 6).

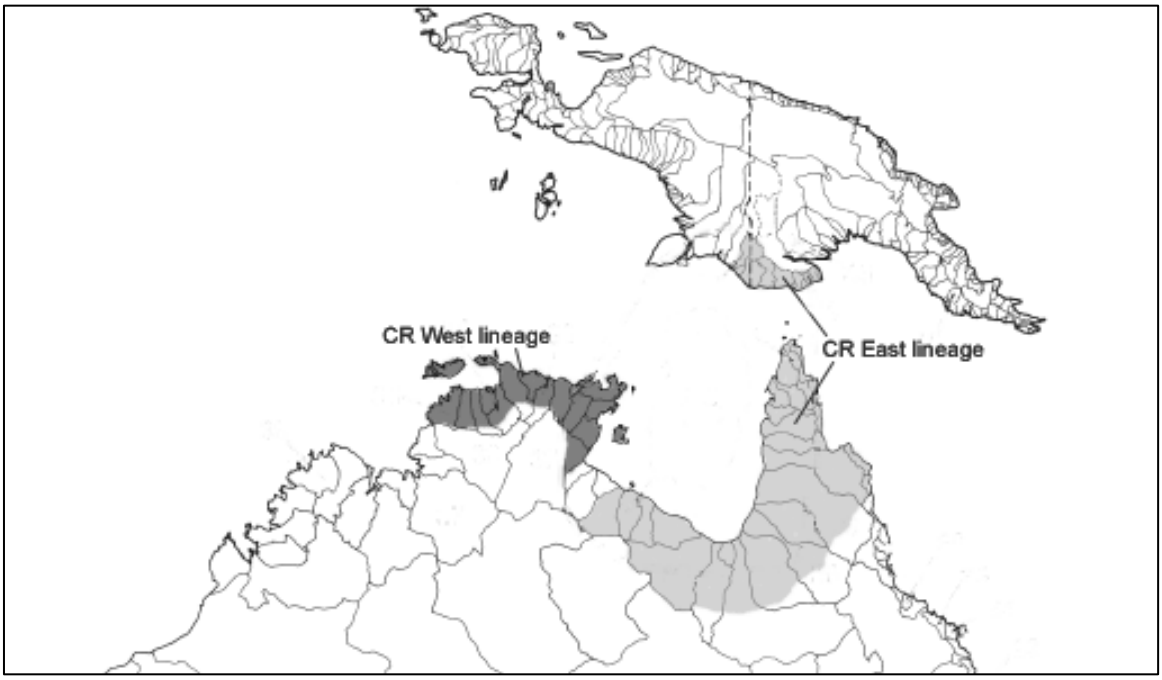

Figure 8.5. Major lineages of *Chelodina rugosa* mapped onto the major drainage basins of Australia, New Guinea and West Papua based on Maximum Parsimony and Maximum Likelihood analyses of 867-bp from the mitochondrial ND4 and control region. (Figure taken from Chapter 6).

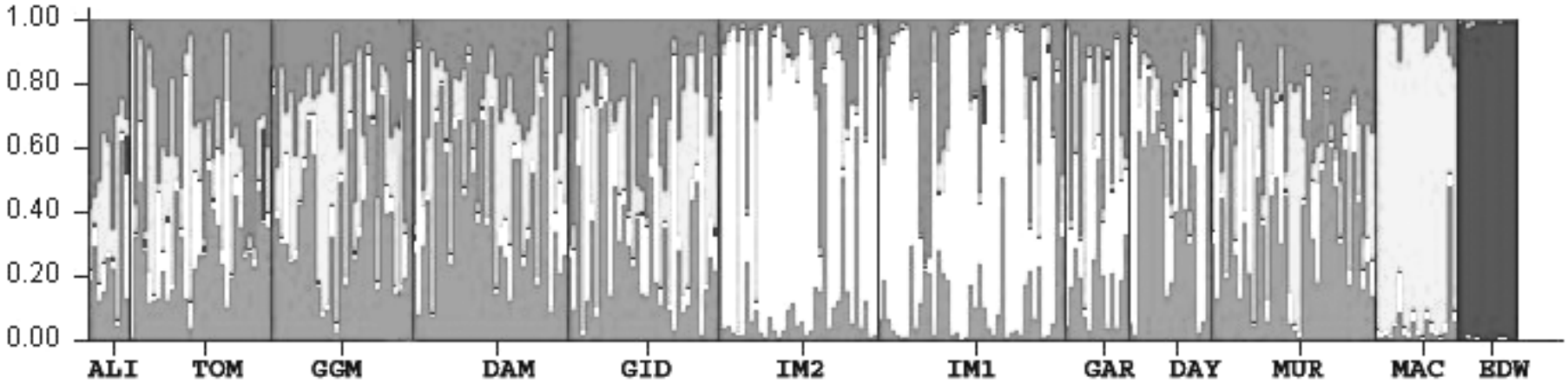

Figure 8.6. A Q-plot for the posterior probability of each individual belonging to five clusters (i.e. populations) that were identified by the admixture model in the Structure analysis, in which: ☐ is cluster1, ☐ is cluster 2, ▒ is cluster 3, ▒ is cluster 4, and ■ is cluster 5.

Populations labelled ALI, TOM, GGM, DAM, GID, IM2, IM1, GAR, DAY, MUR are from the Arnhem Land Plateau in the Northern Territory and individuals could not be assigned to any one cluster (i.e. they have mixed ancestry from clusters 1, 2 and 4). The population labelled MAC is from the Gulf of Carpentaria and all individuals sampled, except for one, were assigned to cluster 3 with high posterior probabilities. All individuals from EDW assigned with high probability to cluster 5.

Two other haplotypes from the *C. burrungandjii* mitochondrial lineage had high posterior probabilities of being pure *C. burrungandjii* (>97%). All the other 55 samples had *C. rugosa* morphology and had high posterior probabilities of being pure *C. rugosa* (>97%). Overall those results suggest that hybridisation and gene flow between the species (i.e. genetic introgression) has been extensive for *C. burrungandjii* and *C. rugosa* of the Northern Territory and only recent hybrids were reliably detected using the 17 microsatellite loci.

Population identification using microsatellites

Bayesian-based clustering algorithms implemented in the program Structure v2.2 detected five clusters across all sampled populations. Individuals from the East Alligator (ALI), Thompkinson (TOM), and Blyth-Cadel basins could not be assigned to a particular cluster and had approximately equal probability of belonging to any of three clusters (i.e. clusters 1, 2 and 4; Figure 8.6). In contrast, all individuals of the MacArthur population, with the exception of one, had a high posterior probability of belonging to cluster 3 (Figure 8.6). Similarly all individuals from the Edward River population had high posterior probabilities of belonging to cluster 5. These results suggest that individuals can be accurately assigned to the MacArthur (Gulf of Carpentaria) and Edward River (Cape York) populations but not to a specific population within the Northern Territory.

In comparison to Structure analyses, GeneClass performed slightly poorer in the correct assignment of individuals to their source population. Approximately 97.4% (294/302) and 97% (293/302) of individuals were accurately assigned to their basin of origin (i.e. the Blyth-Cadel, MacArthur and Edward basins) for Bayesian-based and frequency-based simulations respectively. Similarly to Structure, Geneclass was unable to assign individuals to their source population within the Blyth-Cadel basin with only 37.12% (165/271) and 32.43% (134/271) of individuals correctly assigned for Bayesian- and frequency-based simulations respectively.

Discussion

Species identification and hybridisation.

Application of phylogenetic methods for species identification has the advantage that they are supported by evolutionary theory that describes the ancestral relationships between species in the form of a phylogenetic tree (Avise and Wollenberg 1997). Bootstrap values

on the nodes of the tree provide a level of confidence for correct species identification that can aide in the validation of the phylogenetic data as admissible evidence for court cases (Bernard *et al.* 2007; Rohilla and Tiwari 2008; Salas *et al.* 2007). High bootstrap support across the nodes of the nuclear R35 intron phylogenetic tree indicates that it is a robust method for identification of chelid turtles, albeit not at the species level for some taxon (Figure 8.4). The R35 nuclear intron lacked the resolution to distinguish between all species of Australasian chelids with some species sharing the same haplotype. Paraphyly of the *Elseya* genus (Georges and Adams 1996; Georges *et al.* 1998) and the two major clades of *Chelodina* (Georges *et al.* 2002) for the R35 intron phylogenetic tree (Figure 6.6) supported previous molecular studies (Georges and Adams 1996; Georges *et al.* 1998). Although nuclear markers typically have less resolution compared to mitochondrial DNA for species identification, as observed within the Chelidae family, they can be important markers if hybridisation between species is know to occur, as in *Chelodina* (Pacheo *et al.* 2002; Palumbi and F 1998).

In this study, nuclear markers were used because instances of hybridisation involving (i) *C. rugosa* and *C. burrungandjii*, and (ii) *C. rugosa* and *C. canni* were demonstrated by sequencing of two mitochondrial genes (control region and ND4) and genotyping for 17 microsatellite loci. Mitochondrial *C. burrungandjii* haplotypes from the South Alligator, Cadel and Roper Rivers were closely related to the maternal *C. rugosa* mitochondrial haplotype but had the *C. burrungandjii* morphology. Microsatellites confirmed hybridisation in the mitochondrial *C. burrungandjii* haplotype lineage by identifying an F2 hybrid and two samples that had weak signatures of backcrossing (24% and 22% probabilities respectively), in which fertile hybrids mated with male *C. burrungandjii*. Two other samples had high probabilities of being 'pure' *C. burrungandjii*. Microsatellite results indicate that the hybridisation is both contemporary (hence the F2 hybrid) and historic (hence the weak signatures of backcrossing and 'pure' *C. burrungandjii*). Gene flow between the two species (i.e. introgression) has been extensive based on the absence of a 'pure' *C. burrungandjii* mitochondrial haplotype and the microsatellite results. For the nuclear R35 intron, one of these *C. burrungandjii* mitochondrial haplotypes was correctly identified as *Chelodina burrungandjii*. In the absence of the R35 intron, this sample may have been incorrectly identified as *C. rugosa* based on the mitochondrial sequences alone. Indeed the identification of hybrids is a problem inherent in species identification systems

that use mitochondrial genes in the absence of nuclear markers, including for example the COI barcoding approaches (Hickerson *et al.* 2006; Nelson *et al.* 2007; Vences *et al.* 2005).

Natural introgressive hybridisation between species is a challenge for the forensic identification of seizures because species boundaries become blurred at hybrid contact zones and hybrid individuals can exhibit a complex mixture of morphological and genetic characters inherited from both parental species. Interspecific hybridization and introgression is a common phenomena occurring across all taxonomic groups including plants (e.g. Carlson and Meinke 2008; Repplinger *et al.* 2007; Williams *et al.* 2008; Zha *et al.* 2008), insects (e.g. Beukeboom and van den Assem 2002; Ferris *et al.* 1993; Rasplus *et al.* 2001), mammals (e.g. Adams *et al.* 2007; Ganem *et al.* 2008; Kovacs *et al.* 1997; Randi 2008; Shchipanov *et al.* 2008), birds (e.g. Adamik and Bures 2007; Aliabadian *et al.* 2007; Qvarnstrom *et al.* 2006; Solberg *et al.* 2006) and reptiles (e.g. Cole *et al.* 2007; Leache and Cole 2007; Mebert 2008; Stuart and Parham 2007). A challenge for wildlife forensics is to reliably detect hybrids even if the hybridisation event occurred more than two generations ago. For this study, a combination of two mitochondrial genes, one nuclear gene, and 17 microsatellite markers were able to reliably detect only very recent hybrids (i.e. F2 hybrids) between *C. burrungandjii* and *C. rugosa*.

Emerging molecular technologies such as single nucleotide polymorphisms (SNPs) show great promise for the identification of hybrids. Genotyping a panel of SNPs that are diagnostic at the species-level is an efficient screening method to detect natural hybridisation and introgression. Five species-specific SNPs (both nuclear and chloroplast SNPs) were used to detect natural hybridisation and introgression between *Populus deltoides* and *P. balsamifera* (Hamzeh *et al.* 2007; Meirmans *et al.* 2007). SNP discovery for wildlife species, in which there is typically little to no prior genetic knowledge can be time consuming and laborious. The advent of pyrosequencing technologies is an exciting development that will enable the rapid discovery and genotyping of species-specific SNPs for wildlife (Fakhrai-Rad *et al.* 2002; Novaes *et al.* 2008; Satkoski *et al.* 2008). These technologies are suitable for rapid and large-scale screening of seizures to identify wildlife species and their hybrids.

Regional identification of Chelodina rugosa

There were two distinct mitochondrial lineages for *Chelodina rugosa* that differed by 2.4% sequence divergence and supported the allozyme results of Georges *et al.* (2002). The first lineage included specimens from MacArthur River in the Gulf of Carpentaria and extended eastward to Cape York and included southern New Guinea (East lineage). The second lineage included specimens from Darwin (i.e. Finnis basin), and across Arnhem Land to the Roper basin of the Northern Territory (West lineage). Very high bootstrap support for these two lineages (100 for both MP and ML) indicates that specimens can be reliably identified to either of these two regions demarcated by the two mitochondrial lineages (Figure 8.5) with very high confidence approaching 100%. Within the east lineage there was no phylogeographic structuring as several haplotypes were shared across river basins, thus we can not identify specimens of the East lineage to a specific river. The West lineage, however, revealed phylogeographic structuring in which specimens from the Darwin region (i.e. Finnis basin) formed a clade that was sister to the clade of Arnhem Land haplotypes (i.e. East Alligator, South Alligator, Cadel, Goyder, and Roper) and was supported by high bootstrap support of 99% and 95% for MP and ML respectively. Hence, specimens from Arnhem Land can be distinguished from those of the Darwin region with high levels of confidence. Phylogeographic data are a generally accepted technique that has been used to determine the geographic origins of animals for forensic application. Such data have been used for example, often in conjunction with microsatellite data, to track the geographic origins of poached macaws (Faria *et al.* 2008), wolves (Jedrzejewksi *et al.* 2005), deer (Travis and Keim 1995), seahorses (Sanders *et al.* 2008) and ivory from African elephants (Comstock *et al.* 2003; Nyakaana and Arctander 1999).

Population identification of Chelodina rugosa

Population assignment tests based on 12 microsatellite loci could not correctly assign individuals to their source populations within the Arnhem Land region using either the bayesian- and frequency-based simulations, or clustering approaches. Population assignment tests generally perform poorly when there are insufficient loci sampled, high levels of gene flow between populations or not all populations have been sampled (Cornuet *et al.* 1999a; Manel *et al.* 2002; Manel *et al.* 2005; Waples and Gaggiotti 2006). Given the high levels of correct assignment for individuals from the MacArthur (Gulf of Carpentaria) and Edward rivers (Cape York) for the both simulation and clustering approaches it is

unlikely that the poor assignment of individuals in the Blyth-Cadel basin was a result of inadequate sampling of loci.

High levels of gene flow between populations of the Blyth-Cadel and Liverpool basins were found in Chapter 6, suggesting that they are functioning as a large metapopulation. Furthermore, there are more than 80 sites in the Blyth-Cadel basin alone where *C. rugosa* have been documented to occur (Fordham unpublished data) indicating that they are widely distributed in the basin. This study sampled only eight sites within this region and thus it is highly likely that sites from which individuals may have immigrated from are missing; substantially reducing the power to correctly assign individuals to their source population.

This study highlights one of the major challenges for the identification of wildlife specimens to their population of origin. The resolution to which specimens can be identified to their geographic origins will depend on the dispersal capabilities and population genetic structuring of the species. For species that have excellent dispersal capability and are panmictic, in which there is no genetic structure across their range − sometimes spanning continents (e.g. albatrosses: Burg and Croxall 2001; dolphinfish: Pla and Pujolar 1999) − the assignment of seizures to a specific geographic region will not be possible. On the other end of the spectrum, for species that have poor dispersal capability and isolated populations (e.g. Cabe *et al.* 2007; Schweizer *et al.* 2007; Zamudio and Wieczorek 2007) the seizures can be assigned to very specific localities. *Chelodina rugosa* is intermediate in this spectrum in that specimens were not assigned to a specific site within the Blyth-Cadel basin but were correctly assigned to the Arnhem Land region in the Northern Territory using a combination of both mitochondrial and microsatellite analysis. Future studies for *C. rugosa* could target sites at a scale that is appropriate to identify individuals from different river basins in the Arnhem Land region.

Conclusions

This study adopted an hierarchical approach for a DNA-based system to identify wildlife specimens to species, to regional origin and to population origins. The DNA-based identification system was developed specifically to monitor trade activities, both legal and illegal, of the Australasian snake-necked, *Chelodina rugosa*. The nuclear R35 intron had the appropriate level of resolution to identify *C. rugosa* to the species level, and can

reliably distinguish it from the closely related species *C. burrungandjii*. In addition, the nuclear gene when used in conjunction with mitochondrial genes can identify hybrid and backcrossed individuals. Once the specimen is identified to be *Chelodina rugosa*, further analysis can be conducted to identify its geographic origin using the mitochondrial control region and ND4 genes. Mitochondrial analysis could not distinguish between specimens of southern New Guinea and northern Queensland but can identify if the specimen was sourced from the Northern Territory, and whether it was from the Darwin or Arnhem Land regions. The assignment of a specimen to Arnhem Land was also supported by the microsatellite data. Further assignment of the specimen to a specific population is not possible with the microsatellite data because of the high levels of gene flow of *C. rugosa* amongst sites in the Arnhem Land region. However, the large-scale commercial trade of *C. rugosa* is currently restricted to Maningrida in Arnhem Land which is a region that has restricted access to Aboriginal people, and hence the ability to identify specimens to the Arnhem Land region may be adequate to confirm the legality of animals from the commercial industry.

Chapter 9 – Synopsis

Picture: A hatchling *C. rugosa* from a commercial industry established in Maningrida (Arnhem Land, Northern Territory) that supplies the Australian pet shop industry. Photo by David Frier.

Chapter 9 – Synopsis

The work described in this thesis was funded by the Australian Federal Police as one of three projects with a broad mandate to explore the application of DNA technologies to the regulation and policing of wildlife trade. As such, there were three major themes to the work: (i) identifying specimens to the species level, as this is the fundamental unit recognised by legislation; (ii) assigning specimens to geographic provenance, or at least, providing DNA approaches that can challenge assertions made by defendants regarding the source of specimens and; (iii) utilising the techniques developed to jointly benefit conservation of the traded species and policing of wildlife trade.

Detecting wildlife smuggling operations that are covert in nature and providing evidence for prosecution of perpetrators are the greatest challenges to policing of the wildlife trade. In developing DNA-based systems to provide evidence for wildlife crime, researchers must consider the provisions for accepting scientific data and theory as evidence that were defined by the landmark case of Daubert v Merell Dow Pharmaceuticals US 570 (1993). Evidence must be based on the scientific method and should be: (i) empirically tested, in that the theory or technique is falsifiable, refutable and testable; (ii) subjected to peer review and publication; (iii) known or potential error is quantifiable; and (iv) the theory or technique is generally accepted by the relevant scientific community.

DNA technologies lend themselves to the provision of such evidence and have contributed greatly to our ability to solve crime and re-evaluate earlier convictions. Application to wildlife crime is still in its infancy, even in the United States, where wildlife forensics is arguably best developed. In developing the emerging discipline, one challenge is to draw upon the understanding and strengths of accepted science in the fields of phylogenetics, phylogeography and population genetics and apply this understanding in the areas of species identification and assignment of seized specimens to a region of origin. In this thesis, I have attempted to meet this challenge in the context of a case study of the freshwater turtle *Chelodina rugosa*. As such, this thesis makes contributions both to the discipline of wildlife forensics and to the application of phylogeny, phylogeography and population genetics of *Chelodina rugosa*.

Identifying specimens to the species level

Legislation typically applies to the species as the fundamental unit, and so well defined species boundaries are critical in the development of systems for species identification of wildlife. Species boundaries can become blurred because of unresolved taxonomies or from inter-species hybridisation and introgression. In addition, species concepts vary considerably among scientists and are a topic of much ongoing debate in the literature (Avise and Wollenberg 1997; George and Mayden 2005; Goldstein *et al.* 2005; Mallet 1995; Sites and Marshall 2003; 2004; Templeton 2001). This leads to uncertainty and apparent confusion that could be used to contest the acceptance of evidence in accordance with the Daubert provision (iv) stated above. A consensus needs to be reached by the forensic community as to which species concept to adopt, or at the very least, to clearly state the species concept that is being used in developing evidence.

Taxonomies can be resolved using phylogenetics and phylogeography (Avise 2000; Avise and Wollenberg 1997; Nei and Kumar 2000; Sites and Marshall 2003). In Chapter 6, I used phylogeography to resolve taxonomic issues germane to *Chelodina rugosa*. I found two distinct mitochondrial haplotype clades for *C. rugosa*. I do not suggest that these two haplotype clades represent two biological species; however mtDNA has been used as the sole basis for species designations in the past and these findings could be used by others to erect a new species. If this were to happen, the first haplotype clade would be renamed as *C. oblonga* because the holotype of *Chelodina oblonga* (Gray 1841) was actually a *C. rugosa* drawn from populations of this clade (Thomson 2000; 2006). The second haplotype clade would retain the name *Chelodina rugosa* because it includes the holotype for *C. rugosa* from Cape York (Ogilby 1890). The inclusion of the New Guinea populations in the second haplotype clade refutes their designation as *C. siebenrocki* and supports a previous allozyme study (Georges *et al.* 2002). Resolving these taxonomic issues contributes to our understanding of species boundaries for *C. rugosa* and informs development of species identification systems for the provision of evidence (Chapter 8).

Inter-species hybridisation and introgression have often been overlooked in bringing DNA technologies to bear on species identification. For example, mitochondrial barcoding approaches have been heralded as the future global standard for assigning biological specimens to the correct species (see http://www.dnabarcodes.org/) but as currently formulated, they can not detect hybrids and are often invariant across closely related

species (Ebach and Holdrege 2005; Gregory 2005; Hickerson *et al.* 2006; Moritz and Cicero 2004; Vences *et al.* 2005; Whitworth *et al.* 2007). Based on the extensive inter-species hybridisation and introgression found between *C. rugosa* and its two sympatric species – *C. burrungandjii* and *C. canni* (Chapter 6) – I selected a nuclear gene for species identification. The nuclear gene R35 successfully discriminated specimens of *C. rugosa* from closely related species (Chapter 8). In addition I used mitochondrial (Chapter 6) and microsatellite markers (developed in Chapter 5) for the detection of recent hybrids. Without the comprehensive phylogeographic study (Chapter 6) inappropriate markers for species identification of *C. rugosa* may have been inadvertently selected. This research clearly demonstrates the need for phylogenetic and phylogeography studies to form the scientific foundation for DNA-based species identification systems for wildlife.

Assigning specimens to geographic provenance

Comparative phylogeography can identify patterns of genetic diversity shared by broad taxonomic groups and thereby inform provenance delineation of wildlife specimens. The phylogeographic study of *C. rugosa* identified a sharing of mitochondrial haplotypes between north-east Queensland and New Guinea (Chapter 6). These same phylogeographic patterns have been observed for several taxa owing to connections between northern Australia and New Guinea during the Pleistocene that enabled the exchange of fauna (De Bruyn *et al.* 2004; Lukoschek *et al.* 2007; McGuigan *et al.* 2000; Messmer *et al.* 2005; Murphy and Austin 2004; Rawlings and Donnellan 2003; Ward *et al.* 2006; Williams *et al.* 2008; Wuster *et al.* 2005). Because of the history of the region, for many taxa (such as *C. rugosa*) we can expect that it will not be possible to discriminate between Australian and New Guinea wildlife specimens. This will, however, depend on the species' timing in its colonisation of Australia and New Guinea and whether they were able to disperse via Lake Carpentaria or the Arafura Sill and Torres Strait land bridges.

At a finer scale, population genetic approaches can be used to identify the source of forensic specimens to a specific population (Manel *et al.* 2002). The population genetic study for *C. rugosa* specimens flagged a major challenge for forensics. The resolution to which the geographic origin of a specimen can be identified to is highly dependent on the biology and ecology of the species, and the conduciveness of the landscape to dispersal. The species' local distribution and abundance, dispersal capability, and ability to successfully establish in new sites will drive the patterns of genetic structure observed

across the landscape. Significant levels of genetic structure are required for the correct assignment of specimens to a geographic locality. For highly mobile species, such as *C. rugosa* (Chapters 6 and 8), it may not be possible to identify specimens to a specific population. However, for *C. rugosa* I was able to correctly identify specimens from Arnhem Land and this may adequate to verify the legality of specimens from a commercial industry in Maningrida.

Versatile techniques for conservation and forensic application

Throughout my thesis, I have demonstrated that techniques used to address taxonomic, hybridisation and conservation issues can be adapted for use in forensic applications. This will allow enforcement agencies to capitalise on the genetic data already available for wildlife and reduce development costs for forensic identification systems. Molecular markers used to resolve taxonomic issues in the genus *Chelodina* were adapted for species identification (Chapter 8). Microsatellite markers developed in Chapter 5 were used for population genetic, hybridisation and forensic studies (Chapters 6, 7, and 8). These markers were also tested for their utility in eight other species of *Chelodina*. At least two future studies, to my knowledge, will benefit from these molecular markers (PhD studies of Kate Hodges from the University of Adelaide] and Olivier Baggiano from Griffith University).

It is not only the techniques that can be applied to both conservation and forensic application but also the genetic data itself. The population genetic data for *C. rugosa* was used jointly to provide guidelines for sustainable harvesting in the Blyth-Cadel River (Chapter 7) and for population assignment of specimens via a simple reanalysis of the data (Chapter 8). Similarly, the phylogeographic data was used to understand the historical vicariance events that have shaped genetic diversity of *C. rugosa* (Chapter 6) and for regional identification of specimens (Chapter 8). This research demonstrates that techniques and genetic data can be used jointly to benefit conservation of traded species and the policing of trade. I strongly encourage forensic biologists to form greater collaborations with conservation biologists to develop new molecular markers for wildlife that have the versatility to benefit both disciplines.

Future directions for Wildlife DNA Forensics.

New technologies on the horizon such as whole genome sequencing, single nucleotide polymorphisms (SNPs) and pyrosequencing technologies will pave the way for a new era in wildlife DNA forensics. They will enable prompt discovery of informative molecular markers suitable for identification of specimens at the species (and their hybrids), regional and population levels (Budowle 2004; Cutler *et al.* 2001; Dalma-Weiszhausz and Murphy 2002; Divne and Allen 2005; Jenkins and Gibson 2002; Kwok 2001; McGaugh *et al.* 2007; Syvanen 1999). They are also amenable to high throughput screening of wildlife seizures (Divne and Allen 2005; Jenkins and Gibson 2002; Syvanen 1999). These technologies will complement rather than replace phylogenetics, phylogeography and population genetic approaches. Genetic data from these studies will still be required to delimit species and characterise genetic diversity at inter- and intra-species levels; thereby providing the science to form the foundation for forensic applications. The well-established techniques and theory of phylogenetics, phylogeography and population genetics will be of benefit to future research in the validation of methods and quantification of error for DNA-based wildlife forensic identification systems. We can anticipate that DNA technologies will become an increasingly important tool in international efforts to fight the burgeoning illegal trade of wildlife.

I assert that it is in the best interests of Australia to treat illegal trade of wildlife as the serious crime that it is. The illegal wildlife trade threatens our biodiversity and poses a serious biosecurity risk via the introduction of pests and disease (Normile 2004; Smith *et al.* 2006). Australia is one of the 25 global hotspots for biodiversity (Myers *et al.* 2000) and agriculture is a major industry accounting for approximately 3% of Gross Domestic Product (GDP; Australian Bureau of Agricultural and Resource Economics). Despite the dangers it presents, penalties for illegal wildlife trade in Australia are usually a mere fine that is only a fraction of what the wildlife is worth on the black market (Chapter 2). This is clearly an opportunity for improvement. However, recent developments are positive. The Australian Wildlife Forensic Network (AWFN) was established in 2007 to support, educate and provide evidence for wildlife crime in Australia. The Australian Federal Police has shown the initiative to support this research and the PhD studies of Jo Lee and Linzi Wilson Wilde. These initiatives to improve policing of wildlife crime in Australia need to continue to protect our unique biodiversity and vulnerable agricultural industry.

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